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Novel plant acyltransferases specific for long-chain polyunsaturated fatty acids

The present invention relates to a process for the production of long-chain polyunsaturated fatty acids in an organism by introducing, into the organism, nucleic acids which code for polypeptides with acyltransferase activity. These nucleic acid sequences, if appropriate together with further nucleic acid sequences which code for polypeptides of the fatty acid or lipid metabolism biosynthesis, can advantageously be expressed in the organism. Furthermore, the invention relates to a method for the production of oils and/or triacylglycerides with an elevated content of long-chain polyunsaturated fatty acids.

The invention furthermore relates to the nucleic acid sequences, nucleic acid constructs, vectors and organisms comprising the nucleic acid sequences according to the invention, vectors comprising the nucleic acid sequences and/or the nucleic acid constructs and to transgenic organisms comprising the abovementioned nucleic acid sequences, nucleic acid constructs and/or vectors.

A further part of the invention relates to oils, lipids and/or fatty acids produced by the process according to the invention and to their use.

Fatty acids and triacylglycerides have a multiplicity of applications in the food industry, in animal nutrition, in cosmetics and in the pharmacological sector. Depending on whether they are free saturated or unsaturated fatty acids or else triacylglycerides with an elevated content of saturated or unsaturated fatty acids, they are suitable for very different applications. Polyunsaturated ω-3-fatty acids and ω-6-fatty acids are therefore an important constituent in animal and human food. Owing to the present-day composition of human food, an addition of polyunsaturated ω-3-fatty acids, which are preferentially found in fish oils, to the food is particularly important. Thus, for example, polyunsaturated fatty acids such as docosahexaenoic acid (= DHA, $C22:6^{\Delta 4,7,10,13,16,19}$) or eicosapentaenoic acid (= EPA, $C20:5^{\Delta 5,8,11,14,17}$) are added to baby formula to improve the nutritional value. The unsaturated fatty acid DHA is said to have a positive effect on the development of the brain.

Hereinbelow, polyunsaturated fatty acids are referred to as PUFA, PUFAs, LCPUFA or LCPUFAs (**p**oly **u**nsaturated **f**atty **a**cids, **PUFA**, **l**ong **c**hain **p**oly **u**nsaturated **f**atty **a**cids, **LCPUFA**).

The various fatty acids and triglycerides are mainly obtained from microorganisms such as Mortierella and Schizochytrium or from oil-producing plants such as soybean, oilseed rape, algae such as Crypthecodinium or Phaeodactylum and others, where they are obtained, as a rule, in the form of their triacylglycerides (= triglycerides = triglycerols). However, they can also be obtained from animals, such as, for example, fish. The free fatty acids are advantageously prepared by hydrolysis. Higher polyunsaturated fatty acids such as DHA, EPA, arachidonic acid (= ARA, $C20:4^{\Delta 5,8,11,14}$), dihomo-γ-linolenic acid ($C20:3^{\Delta 8,11,14}$) or docosapentaenoic acid (DPA,

C22:5$^{\Delta 7,10,13,16,19}$) can not be isolated from oil crop plants such as oilseed rape, soybean, sunflower or safflower. Conventional natural sources of these fatty acids are fish such as herring, salmon, sardine, redfish, eel, carp, trout, halibut, mackerel, zander or tuna, or algae.

Depending on the intended use, oils with saturated or unsaturated fatty acids are preferred. In human nutrition, for example, lipids with unsaturated fatty acids, specifically polyunsaturated fatty acids, are preferred. The polyunsaturated ω-3-fatty acids are said to have a positive effect on the cholesterol level in the blood and thus on the possibility of preventing heart disease. The risk of heart disease, stroke or hypertension can be reduced markedly by adding these ω-3-fatty acids to the food. Also, ω-3-fatty acids have a positive effect on inflammatory, specifically on chronically inflammatory, processes in association with immunological diseases such as rheumatoid arthritis. They are therefore added to foodstuffs, specifically to dietetic foodstuffs, or are employed in medicaments. ω-6-Fatty acids such as arachidonic acid tend to have a negative effect on these disorders in connection with these rheumatic diseases on account of our usual dietary intake.

ω-3- and ω-6-fatty acids are precursors of tissue hormones, known as eicosanoids, such as the prostaglandins, which are derived from dihomo-γ-linolenic acid, arachidonic acid and eicosapentaenoic acid, and of the thromoxanes and leukotrienes, which are derived from arachidonic acid and eicosapentaenoic acid. Eicosanoids (known as the PG_2 series) which are formed from ω-6-fatty acids generally promote inflammatory reactions, while eicosanoids (known as the PG_3 series) from ω-3-fatty acids have little or no proinflammatory effect.

Owing to the positive characteristics of the polyunsaturated fatty acids, there has been no lack of attempts in the past to make available genes which are involved in the synthesis of fatty acids or triglycerides for the production of oils in various organisms with a modified content of unsaturated fatty acids. Thus, WO 91/13972 and its US equivalent describe a Δ-9–desaturase. WO 93/11245 claims a Δ-15-desaturase and WO 94/11516 a Δ-12-desaturase. Further desaturases are described, for example, in EP–A–0 550 162, WO 94/18337, WO 97/30582, WO 97/21340, WO 95/18222, EP-A–0 794 250, Stukey et al., J. Biol. Chem., 265, 1990: 20144–20149, Wada et al., Nature 347, 1990: 200–203 or Huang et al., Lipids 34, 1999: 649–659. However, the biochemical characterization of the various desaturases has been insufficient to date since the enzymes, being membrane-bound proteins, present great difficulty in their isolation and characterization (McKeon et al., Methods in Enzymol. 71, 1981: 12141–12147, Wang et al., Plant Physiol. Biochem., 26, 1988: 777–792). As a rule, membrane-bound desaturases are characterized by being introduced into a suitable organism which is subsequently analyzed for enzyme activity by analyzing the starting materials and the products. Δ-6–Desaturases are described in WO 93/06712, US 5,614,393, WO 96/21022, WO 00/21557 and WO 99/27111 and the application for the production in transgenic organisms is described in WO 98/46763, WO 98/46764 and WO 98/46765. In this context, the expression of various desaturases and the

formation of polyunsaturated fatty acids are also described and claimed in WO 99/64616 or WO 98/46776. As regards the expression efficacy of desaturases and its effect on the formation of polyunsaturated fatty acids, it must be noted that the expression of a single desaturase as described to date has only resulted in low contents of unsaturated fatty acids/lipids such as, for example, γ-linolenic acid and stearidonic acid. Moreover, a mixture of ω-3- and ω-6-fatty acids was obtained, as a rule.

Especially suitable microorganisms for the production of PUFAs are microalgae such as Phaeodactylum tricornutum, Porphoridium species, Thraustochytrium species, Schizochytrium species or Crypthecodinium species, ciliates such as Stylonychia or Colpidium, fungi such as Mortierella, Entomophthora or Mucor and/or mosses such as Physcomitrella, Ceratodon and Marchantia (R. Vazhappilly & F. Chen (1998) Botanica Marina 41: 553-558; K. Totani & K. Oba (1987) Lipids 22: 1060-1062; M. Akimoto et al. (1998) Appl. Biochemistry and Biotechnology 73: 269-278). Strain selection has resulted in the development of a number of mutant strains of the microorganisms in question which produce a series of desirable compounds including PUFAs. However, the mutation and selection of strains with an improved production of a particular molecule such as the polyunsaturated fatty acids is a time-consuming and difficult process. This is why recombinant methods as described above are preferred whenever possible. However, only limited amounts of the desired polyunsaturated fatty acids such as DPA, EPA or ARA can be produced with the aid of the abovementioned microorganisms, and, depending on the microorganism used, these are generally obtained as fatty acid mixtures of, for example, EPA, DPA and DHA.

The biosynthesis of LCPUFAs and the incorporation of LCPUFAs into membranes or triacylglycerides proceeds via various metabolic pathways (A. Abbadi et al. (2001) European Journal of Lipid Science & Technology 103:106-113). In bacteria such as Vibrio, and microalgae, such as Schizochytrium, malonyl-CoA is converted into LCPUFAs via an LCPUFA-producing polyketide synthase (J.G. Metz et al. (2001) Science 293: 290-293; WO 00/42195; WO 98/27203; WO 98/55625). In microalgae, such as Phaeodactylum, and mosses, such as Physcomitrella, unsaturated fatty acids such as linoleic acid or linolenic acid are converted, in the form of their acyl-CoAs, in a plurality of desaturation and elongation steps to give LCPUFAs (T.K. Zank et al. (2000) Biochemical Society Transactions 28: 654-658). In mammals, the biosynthesis of DHA comprises a chain shortening via beta-oxidation, in addition to desaturation and elongation steps.

In microorganisms and lower plants, LCPUFAs are present either exclusively in the form of membrane lipids, as is the case in Physcomitrella and Phaeodactylum, or in membrane lipids and triacylglycerides, as is the case in Schizochytrium and Mortierella. Incorporation of LCPUFAs into lipids and oils is catalyzed by various acyltransferases and transacylases. These enzymes are already known to carry out the incorporation of saturated and unsaturated fatty acids [A.R. Slabas (2001) J. Plant Physiology 158: 505-513; M. Frentzen (1998) Fett/Lipid 100: 161-166); S. Cases et al. (1998) Proc. Nat.

Acad. Sci. USA 95: 13018-13023]. The acyltransferases are enzymes of the "Kennedy pathway", which are located on the cytoplasmic side of the membrane system of the endoplasmic reticulum, referred to as "ER" hereinbelow. ER membranes may be isolated experimentally as "microsomal fractions" from various organisms [D.S. Knutzon et al. (1995) Plant Physiology 109: 999-1006; S. Mishra & Y. Kamisaka (2001) Biochemistry 355: 315-322; US 5968791]. These ER-bound acyltransferases in the microsomal fraction use acyl-CoA as the activated form of fatty acids. Glycerol-3-phosphate acyltransferase, referred to as GPAT hereinbelow, catalyzes the incorporation of acyl groups at the sn-1 position of glycerol-3-phosphate. 1-Acylglycerol-3-phosphate acyltransferase (E.C. 2.3.1.51), also known as lysophosphatidic acid acyltransferase and referred to as LPAAT hereinbelow, catalyzes the incorporation of acyl groups at the sn-2 position of lysophosphatidic acid, abbreviated as LPA hereinbelow. After dephosphorylation of phosphatidic acid by phosphatidic acid phosphatase, diacylglycerol acyltransferase, referred to as DAGAT hereinbelow, catalyzes the incorporation of acyl groups at the sn-3 position of diacylglycerols. Apart from these Kennedy pathway enzymes, further enzymes capable of incorporating acyl groups from membrane lipids into triacylglycerides are involved in the incorporation of fatty acids into triacylglycerides, namely phospholipid diacylglycerol acyltransferase, referred to as PDAT hereinbelow, and lysophosphatidylcholine acyltransferase, referred to as LPCAT. Other enzymes too, such as lecithin cholesterol acyltransferase (LCAT) can be involved in the transfer of acyl groups from membrane lipids into triacylglycerides.

In WO 98/54302, Tjoelker et al. disclose a human lysophosphatidic acid acyltransferase and its potential use for the therapy of diseases, as a diagnostic, and a method for identifying modulators of the human LPAAT. In WO 98/54303, Leung et al. describe mammalian lysophosphatidic acid acyltransferases. Moreover, Leung et al. disclose a method for screening pharmaceutical compounds for use, for example, in the treatment of inflammations.

Moreover, a multiplicity of acyltransferases with a wide range of enzymatic functions have been described in the literature and patents; thus, for example, WO 98/55632 and WO 93/10241 describe fatty acid alcohol acyltransferases which are involved in wax synthesis. WO 98/55631 describes a DAGAT (diacylglycerol acyltransferase) from Mortierella ramanniana and a wax synthase from jojoba which also has DAGAT activity. Slabas et al. (WO 94/13814) disclose a membrane-bound sn2-specific acyltransferase which has a different selectivity in the incorporation of monounsaturated erucic acid for the sn2 position and thus makes possible an increased erucic acid yield in oilseed rape. WO 96/24674 describes a corresponding enzyme or gene from Limnanthes douglasii. In WO 95/27791, Davies et al. describe LPAATs which are specific for medium-length fatty acids and incorporate these into the sn2 position of triglycerides. Further novel plant acyltransferase sequences which have been found via homology comparisons with sequences from public databases are described by Lassner et al. (WO 00/18889). Information on the specific function of

these acyltransferase sequences or biochemical data on the corresponding enzymes cannot be found in WO 00/18889.

The enzymic activity of an LPCAT was first described in rats [Land (1960) Journal of Biological Chemistry 235: 2233-2237]. A plastidic LPCAT isoform [Akermoun et al. (2000) Biochemical Society Transactions 28: 713-715] and an ER-bound isoform [Tumaney and Rajasekharan (1999) Biochimica et Biophysica Acta 1439: 47-56; Fraser and Stobart, Biochemical Society Transactions (2000) 28: 715-7718] exist in plants. LPCAT is involved in the biosynthesis and transacylation of polyunsaturated fatty acids in animals as well as in plants [Stymne and Stobart (1984) Biochem. J. 223: 305-314; Stymne and Stobart (1987) in 'The Biochemistry of Plants: a Comprehensive Treatise', Vol. 9 (Stumpf, P.K. ed.) pp. 175-214, Academic Press, New York]. An important function of LPCAT or, more generally, of an acyl-CoA:lysophospholipid acyltransferase, referred to as LPLAT hereinbelow, in the ATP-independent synthesis of acyl-CoA from phospholipids has been described by Yamashita et al. (2001; Journal of Biological Chemistry 276: 26745-26752).

Despite a lot of biochemical data, no genes coding for LPCAT have been identified previously. Genes of various other plant acyltransferases have been isolated and are described in WO 00/18889 (Novel Plant Acyltransferases).

Higher plants comprise polyunsaturated fatty acids such as linoleic acid (C18:2) and linolenic acid (C18:3). ARA, EPA and DHA are found not at all in the seed oil of higher plants, or only in traces (E. Ucciani: Nouveau Dictionnaire des Huiles Végétales. Technique & Documentation – Lavoisier, 1995. ISBN: 2-7430-0009-0). It is advantageous to produce LCPUFAs in higher plants, preferably in oil seeds such as oilseed rape, linseed, sunflower and soybean, since large amounts of high-quality LCPUFAs for the food industry, animal nutrition and pharmaceutical purposes may be obtained at low costs in this way. To this end, it is advantageous to introduce into and express in oil seeds genes coding for enzymes of the biosynthesis of LCPUFAs by genetic engineering methods. Said genes code, for example, for Δ-6-desaturase, Δ-6-elongase, Δ-5-desaturase, Δ-5-elongase and Δ-4-desaturase. These genes may advantageously be isolated from microorganisms and lower plants which produce LCPUFAs and incorporate them in the membranes or triacylglycerides. Thus, Δ-6-desaturase genes have already been isolated from the moss Physcomitrella patens and Δ-6-elongase genes have already been isolated from P. patens and the nematode C. elegans.

Transgenic plants which express genes coding for enzymes of LCPUFA biosynthesis are suitable for producing small amounts of these LCPUFAs; however, there is the risk that the latter are incorporated not into triacylglycerides, but into membranes, since the endogenous acyltransferases and transacylases may not recognize LCPUFAs as substrate and, accordingly, do not incorporate them into triacylglycerides. This is undesired for the following reasons: (i) the main lipid fraction in oil seeds are triacylglycerides. This is why, for economical reasons, it is necessary to concentrate

LCPUFAs in triacylglycerides. LCPUFAs which are incorporated into membranes can modify the physical characteristics of the membranes and thus have harmful effects on the integrity and transport characteristics of the membranes and on the stress tolerance of plants.

First transgenic plants which comprise and express genes coding for enzymes of LCPUFA biosynthesis and produce LCPUFAs have been described for the first time, for example, in DE 102 19 203 (process for the production of polyunsaturated fatty acids in plants). However, these plants produce LCPUFAs in amounts which require further optimization for processing the oils present in said plants.

In order to enable food and feed to be enriched with these polyunsaturated fatty acids, there is therefore a great need for a simple, inexpensive process for producing said polyunsaturated fatty acids, especially in eukaryotic systems.

It was therefore the object to develop a process for the production of polyunsaturated fatty acids in an organism, advantageously in a eukaryotic organism, preferably in a plant. This object was achieved by the process according to the invention for the production of polyunsaturated fatty acids in an organism, which comprises the following steps:

a) introducing, into the organism, at least one nucleic acid sequence with the sequence shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18 or SEQ ID NO: 20, which codes for a polypeptide with lysophosphatidic acid acyltransferase activity; or

b) introducing, into the organism, at least one nucleic acid sequence with the sequence shown in SEQ ID NO: 22, SEQ ID NO: 24 or SEQ ID NO: 26, which codes for a polypeptide with glycerol-3-phosphate acyltransferase activity; or

c) introducing, into the organism, at least one nucleic acid sequence with the sequence shown in SEQ ID NO: 28, SEQ ID NO: 30 or SEQ ID NO: 32 which codes for a polypeptide with diacylglycerol acyltransferase activity; or

d) introducing, into the organism, at least one nucleic acid sequence with the sequence shown in SEQ ID NO: 34 or SEQ ID NO: 36, which codes for a polypeptide with lecithin cholesterol acyltransferase activity; or

e) introducing, into the organism, at least one nucleic acid sequence which can be derived from the coding sequence in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 as the result of the degeneracy of the genetic code, or

f) introducing, into the organism, at least one derivative of the nucleic acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36, which code for polypeptides with the amino acid sequence shown in SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35 or SEQ ID NO: 37 and which have at least 40% homology at the amino acid level with SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35 or SEQ ID NO: 37 and have an equivalent lysophosphatidic acid acyltransferase activity, glycerol-3-phosphate acyltransferase activity, diacylglycerol acyltransferase activity or lecithin cholesterol acyltransferase activity, and

g) culturing and harvesting the organism.

Advantageously, the polyunsaturated fatty acids produced in the process of the invention comprise at least two, advantageously three, four or five, double bonds. The fatty acids particularly advantageously comprise four or five double bonds. Fatty acids produced in the process advantageously have 18, 20, 22 or 24 carbon atoms in the fatty acid chain; preferably, the fatty acids comprise 20, 22 or 24 carbon atoms in the fatty acid chain. Advantageously, saturated fatty acids are reacted to a minor extent, or not at all, with the nucleic acids used in the process. A minor extent is understood as meaning that the saturated fatty acids are reacted with less than 5%, advantageously less than 3%, especially advantageously with less than 2% of the activity in comparison with polyunsaturated fatty acids. These fatty acids which are produced may be produced in the process as a single product or be present in a fatty acid mixture.

The nucleic acid sequences used in the process of the invention are isolated nucleic acid sequences which code for polypeptides with lysophosphatidic acid acyltransferase activity, glycerol-3-phosphate acyltransferase activity, diacylglycerol acyltransferase activity and/or lecithin cholesterol acyltransferase activity.

The polyunsaturated fatty acids produced in the process are advantageously bound in membrane lipids and/or triacylglycerides but may also occur in the organisms as free fatty acids or else bound in the form of other fatty acid esters. In this context, they may be present as stated as "pure products" or else advantageously in the form of mixtures of various fatty acids or mixtures of different glycerides. The various fatty acids bound in the triacylglycerides can be derived here from short-chain fatty acids having from 4 to 6 carbon atoms, medium-chain fatty acids having from 8 to 12 carbon atoms or long-chain fatty acids having from 14 to 24 carbon atoms, with preference being given to the

long-chain fatty acids and particular preference being given to the long-chain fatty acids, LCPUFAs, of C_{18}-, C_{20}-, C_{22}- and/or C_{24}-fatty acids.

The process of the invention advantageously produces fatty acid esters with polyunsaturated C_{18}-, C_{20}-, C_{22}- and/or C_{24}-fatty acid molecules, with at least two double bonds being present in the fatty acid ester. These fatty acid molecules preferably comprise three, four or five double bonds and advantageously lead to the synthesis of hexadecadienoic acid (C16:2$^{\Delta 9,12}$), γ-linolenic acid (= GLA, C18:3$^{\Delta 6,9,12}$), stearidonic acid (= SDA, C18:4$^{\Delta 6,9,12,15}$), dihomo-γ-linolenic acid (= DGLA, 20:3$^{\Delta 8,11,14}$), eicosatetraenoic acid (= ETA, C20:4$^{\Delta 5,8,11,14}$), arachidonic acid (ARA), eicosapentaenoic acid (EPA) or mixtures thereof, preferably EPA and/or ARA.

The fatty acid esters with polyunsaturated C_{18}-, C_{20}-, C_{22}- and/or C_{24}-fatty acid molecules can be isolated, from the organisms which have been used for the preparation of the fatty acid esters, in the form of an oil or lipid, for example in the form of compounds such as sphingolipids, phosphoglycerides, lipids, glycolipids such as glycosphingolipid, phospholipids such as phosphatidylethanolamine, phosphatidylcholine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol or diphosphatidylglycerol, monoacylglycerides, diacylglycerides, triacylglycerides or other fatty acid esters such as the acetyl-coenzyme A esters which comprise the polyunsaturated fatty acids with at least two, preferably three double bonds; advantageously they are isolated in the form of their diacylglycerides, triacylglycerides and/or in the form of phosphatidylcholine, especially preferably in the form of the triacylglycerides. In addition to these esters, the polyunsaturated fatty acids are also present in the organisms, advantageously the plants, as free fatty acids or bound in other compounds. As a rule, the various abovementioned compounds (fatty acid esters and free fatty acids) are present in the organisms with an approximate distribution of 80 to 90% by weight of triglycerides, 2 to 5% by weight of diglycerides, 5 to 10% by weight of monoglycerides, 1 to 5% by weight of free fatty acids, 2 to 8% by weight of phospholipids, the total of the various compounds amounting to 100% by weight.

The process according to the invention yields the LCPUFAs produced in a content of at least 3% by weight, advantageously at least 5% by weight, preferably at least 8% by weight, especially preferably at least 10% by weight, most preferably at least 15% by weight, based on the total fatty acids in the transgenic organisms, advantageously in a transgenic plant. The fatty acids are advantageously produced in bound form. With the aid of the nucleic acids used in the process according to the invention, these unsaturated fatty acids can be brought into the sn1, sn2 and/or sn3 position of the triglycerides which are advantageously prepared. Since a plurality of reaction steps are performed by the starting compounds hexadecadienoic acid (C16:2), linoleic acid (C18:2) and linolenic acid (C18:3) in the process according to the invention, the end products of the process such as, for example, arachidonic acid (ARA) or eicosapentaenoic acid (EPA) are not obtained as absolutely pure products; minor traces of the precursors are always present in the end product. If, for example, both linoleic acid and linolenic acid are present in the starting organism and the starting

plant, the end products such as ARA and EPA are present as mixtures. The precursors should advantageously not amount to more than 20% by weight, preferably not to more than 15% by weight, especially preferably not to more than 10% by weight, most preferably not to more than 5% by weight, based on the amount of the end product in question. Advantageously, only ARA or only EPA, bound or as free acids, are produced as end products in a transgenic plant in the process according to the invention. If both compounds (ARA and EPA) are produced simultaneously, they are advantageously produced in a ratio of at least 1:2 (EPA:ARA), advantageously of at least 1:3, preferably 1:4, especially preferably 1:5.

Owing to the nucleic acid sequences according to the invention, an increase in the yield of polyunsaturated fatty acids of at least 50%, advantageously of at least 80%, especially advantageously of at least 100%, very especially advantageously of at least 150%, in comparison with the nontransgenic starting organism, can be obtained by comparison in GC analysis (see examples). In a further advantageous embodiment, the yield of polyunsaturated fatty acids can be increased by at least 200%, preferably by at least 250%, very especially preferably by at least 300%.

Chemically pure polyunsaturated fatty acids or fatty acid compositions can also be synthesized by the processes described above. To this end, the fatty acids or the fatty acid compositions are isolated from the organism, such as the microorganisms or the plants or the culture medium in or on which the organisms have been grown, or from the organism and the culture medium, in the known manner, for example via extraction, distillation, crystallization, chromatography or combinations of these methods. These chemically pure fatty acids or fatty acid compositions are advantageous for applications in the food industry sector, the cosmetics industry sector and especially the pharmacological industry sector.

Suitable organisms for the production in the process according to the invention are, in principle, any organisms such as microorganisms, nonhuman animals or plants. Advantageously the process according to the invention employs transgenic organisms such as fungi, such as Mortierella or Traustochytrium, yeasts such as Saccharomyces or Schizosaccharomyces, mosses such as Physcomitrella or Ceratodon, nonhuman animals such as Caenorhabditis, algae such as Crypthecodinium or Phaeodactylum or plants such as dicotyledonous or monocotyledonous plants. Organisms which are especially advantageously used in the process according to the invention are organisms which belong to the oil-producing organisms, that is to say which are used for the production of oils, such as fungi, such as Mortierella or Traustochytrium, algae such as Crypthecodinium, Phaeodactylum, or plants, in particular plants, preferably oil crop plants which comprise large amounts of lipid compounds, such as peanut, oilseed rape, canola, sunflower, safflower, poppy, mustard, hemp, castor-oil plant, olive, sesame, Calendula, Punica, evening primrose, verbascum, thistle, wild roses, hazelnut, almond, macadamia, avocado, bay, pumpkin/squash, linseed, soybean, pistachios, borage, trees (oil palm, coconut or walnut) or arable crops such as maize, wheat, rye, oats, triticale, rice, barley, cotton, cassava, pepper, Tagetes, Solanaceae plants such

as potato, tobacco, eggplant and tomato, Vicia species, pea, alfalfa or bushy plants (coffee, cacao, tea), Salix species, and perennial grasses and fodder crops. Preferred plants according to the invention are oil crop plants such as peanut, oilseed rape, canola, sunflower, safflower, poppy, mustard, hemp, castor-oil plant, olive, Calendula, Punica, evening primrose, pumpkin/squash, linseed, soybean, borage, trees (oil palm, coconut). Especially preferred are plants which are high in C18:2- and/or C18:3-fatty acids, such as sunflower, safflower, tobacco, verbascum, sesame, cotton, pumpkin/squash, poppy, evening primrose, walnut, linseed, hemp, thistle or safflower. Very especially preferred plants are plants such as safflower, sunflower, poppy, evening primrose, walnut, linseed or hemp.

It is advantageous to the inventive process described to introduce, in addition to the nucleic acids introduced in step (a) to (f) of the process, further nucleic acids which code for enzymes of the fatty acid or lipid metabolism into the organism.

In principle, all genes of the fatty acid or lipid metabolism can be used in the process for the production of polyunsaturated fatty acids, advantageously in combination with the inventive acyl-CoA:lysophospholipid acyltransferase. Genes of the fatty acid or lipid metabolism selected from the group consisting of acyl-CoA dehydrogenase(s), acyl-ACP [= acyl carrier protein] desaturase(s), acyl-ACP thioesterase(s), fatty acid acyltransferase(s), acyl-CoA:lysophospholipid acyltransferases, fatty acid synthase(s), fatty acid hydroxylase(s), acetyl-coenzyme A carboxylase(s), acyl-coenzyme A oxidase(s), fatty acid desaturase(s), fatty acid acetylenases, lipoxygenases, triacylglycerol lipases, alleneoxide synthases, hydroperoxide lyases or fatty acid elongase(s) are advantageously used in combination with the acyl-CoA:lysophospholipid acyltransferase. Genes selected from the group of the acyl-CoA:lysophospholipid acyltransferases, Δ-4-desaturases, Δ-5-desaturases, Δ-6-desaturases, Δ-8-desaturases, Δ-9-desaturases, Δ-12-desaturases, Δ-5-elongases, Δ-6-elongases or Δ-9-elongases are especially preferably used in combination with the abovementioned genes for lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase, it being possible to use individual genes or a plurality of genes in combination.

Owing to the enzymatic activity of the nucleic acids used in the process according to the invention which code for polypeptides with lysophosphatidic acid acyltransferase glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase activity, advantageously in combination with nucleic acid sequences which code for polypeptides of the fatty acid or lipid metabolism, such as the acyl-CoA:lysophospholipid acyltransferase activity, the Δ-4-, Δ-5-, Δ-6-, Δ-8-desaturase or the Δ-5-, Δ-6- or Δ-9-elongase activity, a wide range of polyunsaturated fatty acids can be produced in the process according to the invention. Depending on the choice of the organisms, such as the advantageous plant, used for the process according to the invention, mixtures of the various polyunsaturated fatty acids or individual polyunsaturated fatty acids, such as EPA or ARA, can be produced in free or bound form. Depending on the prevailing fatty acid composition in the starting plant

(C18:2- or C18:3-fatty acids), fatty acids which are derived from C18:2-fatty acids, such as GLA, DGLA or ARA, or fatty acids which are derived from C18:3-fatty acids, such as SDA, ETA or EPA, are thus obtained. If only linoleic acid (= LA, C18:$2^{\Delta 9,12}$) is present as unsaturated fatty acid in the plant used for the process, the process can only afford GLA, DGLA and ARA as products, all of which can be present as free fatty acids or in bound form. If only α-linolenic acid (= ALA, C18:$3^{\Delta 9,12,15}$) is present as unsaturated fatty acid in the plant used for the process, as is the case, for example, in linseed, the process can only afford SDA, ETA and EPA as products, all of which can be present as free fatty acids or in bound form, as described above. By modifying the activity of the enzymes involved in the synthesis, lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase advantageously in combination with acyl-CoA:lysophospholipid acyltransferase, Δ-5-, Δ-6-desaturase and/or Δ-6-elongase or with acyl-CoA:lysophospholipid acyltransferase, Δ-5-, Δ-8-desaturase and/or Δ-9-elongase or in combination with only the first three genes, acyl-CoA:lysophospholipid acyltransferase, Δ-6-desaturase and/or Δ-6-elongase or acyl-CoA:lysophospholipid acyltransferase, Δ-8-desaturase and Δ-9-elongase, of the synthesis cascade, it is possible to produce, in a targeted fashion, only individual products in the abovementioned organisms, advantageously in the abovementioned plants. Owing to the activity of Δ-6-desaturase and Δ-6-elongase, for example, GLA and DGLA, or SDA and ETA, are formed, depending on the starting plant and unsaturated fatty acid. DGLA or ETA or mixtures of these are preferably formed. If Δ-5-desaturase is additionally introduced into the organisms, advantageously into the plant, ARA or EPA is additionally formed. This also applies to organisms into which Δ-8-desaturase and Δ-9-elongase have been introduced previously. Advantageously, only ARA or EPA or mixtures of these are synthesized, depending on the fatty acid present in the organism, or in the plant, which acts as starting substance for the synthesis. Since biosynthetic cascades are involved, the end products in question are not present in pure form in the organisms. Small amounts of the precursor compounds are always additionally present in the end product. These small amounts amount to less than 20% by weight, advantageously less than 15% by weight, especially advantageously less than 10% by weight, most advantageously less than 5, 4, 3, 2 or 1% by weight, based on the end product DGLA, ETA or their mixtures, or ARA, EPA or their mixtures.

To increase the yield in the described method for the production of oils and/or triglycerides with an advantageously elevated content of polyunsaturated fatty acids, it is advantageous to increase the amount of starting product for the synthesis of fatty acids; this can be achieved for example by introducing, into the organism, a nucleic acid which codes for a polypeptide with Δ-12-desaturase. This is particularly advantageous in oil-producing organisms such as oilseed rape which are high in oleic acid. Since these organisms are only low in linoleic acid (Mikoklajczak et al., Journal of the American Oil Chemical Society, 38, 1961, 678 - 681), the use of the abovementioned Δ-12-desaturases for producing the starting material linoleic acid is advantageous.

Nucleic acids used in the process according to the invention are advantageously derived from plants such as algae such as Isochrysis or Crypthecodinium, algae/diatoms such as Phaeodactylum, mosses such as Physcomitrella or Ceratodon, or higher plants such as the Primulaceae such as Aleuritia, Calendula stellata, Osteospermum spinescens or Osteospermum hyoseroides, microorganisms such as fungi, such as Aspergillus, Thraustochytrium, Phytophthora, Entomophthora, Mucor or Mortierella, bacteria such as Shewanella, yeasts or animals such as nematodes such as Caenorhabditis, insects or humans. The nucleic acids are advantageously derived from fungi, animals, or from plants such as algae or mosses, preferably from nematodes such as Caenorhabditis.

The process according to the invention advantageously employs the abovementioned nucleic acid sequences or their derivative or homologs which code for polypeptides which retain the enzymatic activity of the proteins encoded by nucleic acid sequences. These sequences, individually or in combination with the nucleic acid sequences which code for lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase and/or lecithin cholesterol acyltransferase are cloned into expression constructs and used for the introduction into, and expression in, organisms. Owing to their construction, these expression constructs make possible an advantageous optimal synthesis of the polyunsaturated fatty acids produced in the process according to the invention.

In a preferred embodiment, the process furthermore comprises the step of obtaining a cell or an intact organism which comprises the nucleic acid sequences used in the process, where the cell and/or the organism is transformed with a nucleic acid sequence according to the invention which codes for the lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase and/or lecithin cholesterol acyltransferase, a gene construct or a vector as described below, alone or in combination with further nucleic acid sequences which code for proteins of the fatty acid or lipid metabolism. In a further preferred embodiment, this process furthermore comprises the step of obtaining the fine chemical from the culture. The culture can, for example, take the form of a fermentation culture, for example in the case of the cultivation of microorganisms, such as, for example, Mortierella, Saccharomyces or Traustochytrium, or a greenhouse- or field-grown culture of a plant. The cell or the organism produced thus is advantageously a cell of an oil-producing organism, such as an oil crop plant, such as, for example, peanut, oilseed rape, canola, linseed, hemp, soybean, safflower, sunflowers or borage.

In the case of plant cells, plant tissue or plant organs, "growing" is understood as meaning, for example, the cultivation on or in a nutrient medium, or of the intact plant on or in a substrate, for example in a hydroponic culture, potting compost or on arable land.

For the purposes of the invention, "transgenic" or "recombinant" means, with regard to the example of a nucleic acid sequence, an expression cassette (= gene construct) or a

vector comprising the nucleic acid sequence according to the invention or an organism transformed with the nucleic acid sequences, expression cassette or vector according to the invention, all those constructions brought about by recombinant methods in which either

a) the nucleic acid sequence according to the invention, or

b) a genetic control sequence which is operably linked with the nucleic acid sequence according to the invention, for example a promoter, or

c) (a) and (b)

are not located in their natural genetic environment or have been modified by recombinant methods, it being possible for the modification to take the form of, for example, a substitution, addition, deletion, inversion or insertion of one or more nucleotide residues. The natural genetic environment is understood as meaning the natural genomic or chromosomal locus in the original organism or the presence in a genomic library. In the case of a genomic library, the natural genetic environment of the nucleic acid sequence is preferably retained, at least in part. The environment flanks the nucleic acid sequence at least on one side and has a sequence length of at least 50 bp, preferably at least 500 bp, especially preferably at least 1000 bp, most preferably at least 5000 bp. A naturally occurring expression cassette – for example the naturally occurring combination of the natural promoter of the inventive nucleic acid sequences with the corresponding lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase and/or lecithin cholesterol acyltransferase genes - becomes a transgenic expression cassette when this expression cassette is modified by nonnatural, synthetic ("artificial") methods such as, for example, mutagenic treatment. Suitable methods are described, for example, in US 5,565,350 or WO 00/15815.

A transgenic organism or transgenic plant for the purposes of the invention is understood as meaning, as above, that the nucleic acids used in the process are not at their natural locus in the genome of an organism, it being possible for the nucleic acids to be expressed homologously or heterologously. However, as mentioned, transgenic also means that, while the nucleic acids according to the invention are at their natural position in the genome of an organism, the sequence has been modified with regard to the natural sequence, and/or that the regulatory sequences of the natural sequences have been modified. Transgenic is preferably understood as meaning the expression of the nucleic acids according to the invention at an unnatural locus in the genome, i.e. homologous or, preferably, heterologous expression of the nucleic acids takes place. Preferred transgenic organisms are fungi such as Mortierella, mosses such as Physcomitrella, algae such as Cryptocodinium or plants such as the oil crop plants.

Suitable organisms or host organisms for the nucleic acids, the expression cassette or the vector used in the process according to the invention are, in principle, advantageously all organisms which are capable of synthesizing fatty acids, specifically

unsaturated fatty acids, and/or which are suitable for the expression of recombinant genes. Examples which may be mentioned are plants such as Arabidopsis, Asteraceae such as Calendula or crop plants such as soybean, peanut, castor-oil plant, sunflower, maize, cotton, flax, oilseed rape, coconut, oil palm, safflower (Carthamus tinctorius) or cacao bean, microorganisms, such as fungi, for example the genus Mortierella, Thraustochytrium, Saprolegnia, or Pythium, bacteria, such as the genus Escherichia, or Shewanella, yeasts, such as the genus Saccharomyces, cyanobacteria, ciliates, algae or protozoans such as dinoflagellates, such as Crypthecodinium. Preferred organisms are those which are naturally capable of synthesizing substantial amounts of oil, such as fungi, such as Mortierella alpina, Pythium insidiosum, or plants such as soybean, oilseed rape, coconut, oil palm, safflower, flax, hemp, castor-oil plant, Calendula, peanut, cacao bean or sunflower, or yeasts such as Saccharomyces cerevisiae, with soybean, flax, oilseed rape, safflower, sunflower, Calendula, Mortierella or Saccharomyces cerevisiae being especially preferred. In principle, suitable host organisms are, in addition to the abovementioned transgenic organisms, also transgenic animals, advantageously nonhuman animals, for example C. elegans.

Further utilizable host cells are detailed in: Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990).

Expression strains which can be used, for example those with a lower protease activity, are described in: Gottesman, S., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 119-128.

These include plant cells and certain tissues, organs and parts of plants in all their phenotypic forms such as anthers, fibers, root hairs, stalks, embryos, calli, cotyledons, petioles, harvested material, plant tissue, reproductive tissue and cell cultures which are derived from the actual transgenic plant and/or can be used for giving rise to the transgenic plant.

Transgenic plants which comprise the polyunsaturated fatty acids synthesized in the process according to the invention can advantageously be marketed directly without there being any need for the oils, lipids or fatty acids synthesized to be isolated. Plants for the process according to the invention are listed as meaning intact plants and all plant parts, plant organs or plant parts such as leaf, stem, seeds, root, tubers, anthers, fibers, root hairs, stalks, embryos, calli, cotyledons, petioles, harvested material, plant tissue, reproductive tissue and cell cultures which are derived from the transgenic plant and/or can be used for giving rise to the transgenic plant. In this context, the seed comprises all parts of the seed such as the seed coats, epidermal cells, seed cells, endosperm or embryonic tissue. However, the compounds produced in the process according to the invention can also be isolated from the organisms, advantageously plants, in the form of their oils, fat, lipids and/or free fatty acids. Polyunsaturated fatty acids produced by this process can be obtained by harvesting the organisms, either from the crop in which they grow, or from the field. This can be done via pressing or extraction of the plant parts, preferably the plant seeds. In this context, the oils, fats,

lipids and/or free fatty acids can be obtained by what is known as cold-beating or cold-pressing without applying heat by pressing. To allow for greater ease of disruption of the plant parts, specifically the seeds, they are previously comminuted, steamed or roasted. The seeds which have been pretreated in this manner can subsequently be pressed or extracted with solvents such as warm hexane. The solvent is subsequently removed again. In the case of microorganisms, the latter are, after harvesting, for example extracted directly without further processing steps or else, after disruption, extracted via various methods with which the skilled worker is familiar. In this manner, more than 96% of the compounds produced in the process can be isolated. Thereafter, the resulting products are processed further, i.e. refined. In this process, substances such as the plant mucilages and suspended matter are first removed. What is known as desliming can be effected enzymatically or, for example, chemico-physically by addition of acid such as phosphoric acid. Thereafter, the free fatty acids are removed by treatment with a base, for example sodium hydroxide solution. The resulting product is washed thoroughly with water to remove the alkali remaining in the product and then dried. To remove the pigments remaining in the product, the products are subjected to bleaching, for example using fuller's earth or active charcoal. At the end, the product is deodorized, for example using steam.

The PUFAs or LCPUFAs produced by this process are preferably C_{18}-, C_{20}-, C_{22}- or C_{24}-fatty acid molecules with at least two double bonds in the fatty acid molecule, preferably three, four, five or six double bonds. These C_{18}-, C_{20}-, C_{22}- or C_{24}-fatty acid molecules can be isolated from the organism in the form of an oil, a lipid or a free fatty acid. Suitable organisms are, for example, those mentioned above. Preferred organisms are transgenic plants.

One embodiment of the invention is therefore oils, lipids or fatty acids or fractions thereof which have been produced by the above-described process, especially preferably oil, lipid or a fatty acid composition comprising PUFAs and being derived from transgenic plants.

A further embodiment according to the invention is the use of the oil, lipid, the fatty acids and/or the fatty acid composition in feedstuffs, foodstuffs, cosmetics or pharmaceuticals.

The term "oil", "lipid" or "fat" is understood as meaning a fatty acid mixture comprising unsaturated or saturated, preferably esterified, fatty acid(s). The oil, lipid or fat is preferably high in polyunsaturated free or, advantageously, esterified fatty acid(s), in particular linoleic acid, γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, α-linolenic acid, stearidonic acid, eicosatetraenoic acid, eicosapentaenoic acid, docosapentaenoic acid or docosahexaenoic acid. The content of unsaturated esterified fatty acids preferably amounts to approximately 30%, a content of 50% is more preferred, a content of 60%, 70%, 80% or more is even more preferred. For the analysis, the fatty acid content can, for example, be determined by gas chromatography after converting the fatty acids into the methyl esters by transesterification. The

oil, lipid or fat can comprise various other saturated or unsaturated fatty acids, for example calendulic acid, palmitic acid, palmitoleic acid, stearic acid, oleic acid and the like. The content of the various fatty acids in the oil or fat can vary in particular, depending on the starting organism.

The polyunsaturated fatty acids with advantageously at least two double bonds which are produced in the process are, as described above, for example sphingolipids, phosphoglycerides, lipids, glycolipids, phospholipids, monoacylglycerol, diacylglycerol, triacylglycerol or other fatty acid esters.

Starting from the polyunsaturated fatty acids with advantageously at least two double bonds, which acids have been prepared in the process according to the invention, the polyunsaturated fatty acids which are present can be liberated for example via treatment with alkali, for example aqueous KOH or NaOH, or acid hydrolysis, advantageously in the presence of an alcohol such as methanol or ethanol, or via enzymatic cleavage, and isolated via, for example, phase separation and subsequent acidification via, for example, H_2SO_4. The fatty acids can also be liberated directly without the above-described processing step.

After their introduction into an organism, advantageously a plant cell or plant, the nucleic acids used in the process can either be present on a separate plasmid or integrated into the genome of the host cell. In the case of integration into the genome, integration can be random or else be effected by recombination such that the native gene is replaced by the copy introduced, whereby the production of the desired compound by the cell is modulated, or by the use of a gene in trans, so that the gene is linked functionally with a functional expression unit which comprises at least one sequence which ensures the expression of a gene and at least one sequence which ensures the polyadenylation of a functionally transcribed gene. The nucleic acids are advantageously introduced into the organisms via multiexpression cassettes or constructs for multiparallel expression, advantageously into the plants for the multiparallel seed-specific expression of genes.

Mosses and algae are the only known plant systems which produce substantial amounts of polyunsaturated fatty acids such as arachidonic acid (ARA) and/or eicosapentaenoic acid (EPA) and/or docosahexaenoic acid (DHA). Mosses comprise PUFAs in membrane lipids, while algae, organisms which are related to algae and a few fungi also accumulate substantial amounts of PUFAs in the triacylglycerol fraction. This is why nucleic acid molecules are suitable which are isolated from such strains which also accumulate PUFAs in the triacylglycerol fraction, particularly advantageously for the process according to the invention and thus for the modification of the lipid and PUFA production system in a host, in particular plants such as oil crop plants, for example oilseed rape, canola, linseed, hemp, soybeans, sunflowers and borage. They can therefore be used advantageously in the process according to the invention.

Substrates of the nucleic acids used in the process according to the invention which code for polypeptides with lysophosphatidic acid acyltransferase activity, glycerol-3-phosphate acyltransferase activity, diacylglycerol acyltransferase activity or lecithin cholesterol acyltransferase activity, and/or of the further nucleic acids used, such as the nucleic acids which code for polypeptides of the fatty acid metabolism or lipid metabolism selected from the group consisting of acyl-CoA dehydrogenase(s), acyl-ACP[= acyl carrier protein] desaturase(s), acyl–ACP thioesterase(s), fatty acid acyltransferase(s), acyl-CoA:lysophospholipid acyltransferase(s), fatty acid synthase(s), fatty acid hydroxylase(s), acetyl coenzyme A carboxylase(s), acyl coenzyme A oxidase(s), fatty acid desaturase(s), fatty acid acetylenase(s), lipoxygenase(s), triacylglycerol lipase(s), allene oxide synthase(s), hydroperoxide lyase(s) or fatty acid elongase(s) which are advantageously suitable are C_{16}-, C_{18}-, C_{20}- or C_{22}-fatty acids. The fatty acids converted in the process in the form of substrates are preferably converted in the form of their acyl-CoA esters.

To produce the long-chain PUFAs according to the invention, the polyunsaturated C_{16}- or C_{18}-fatty acids must first be desaturated by the enzymatic activity of a desaturase and subsequently be elongated by at least two carbon atoms via an elongase. After one elongation cycle, this enzyme activity gives C_{18}- or C_{20}-fatty acids and after two or three elongation cycles C_{22}- or C_{24}-fatty acids. The activity of the desaturases and elongases used in the process according to the invention preferably leads to C_{18}-, C_{20}-, C_{22}- and/or C_{24}-fatty acids, advantageously with at least two double bonds in the fatty acid molecule, preferably with three, four or five double bonds, especially preferably to give C_{20}- and/or C_{22}-fatty acids with at least two double bonds in the fatty acid molecule, preferably with three, four or five double bonds in the molecule. After a first desaturation and the elongation have taken place, further desaturation steps such as, for example, one in the $\Delta 5$ position may take place. Products of the process according to the invention which are especially preferred are dihomo-γ-linolenic acid, arachidonic acid, eicosapentaenoic acid, docosapentaenoic acid and/or docosahexaenoic acid. The C_{18}-fatty acids with at least two double bonds in the fatty acid can be elongated by the enzymatic activity according to the invention in the form of the free fatty acid or in the form of the esters, such as phospholipids, glycolipids, sphingolipids, phosphoglycerides, monoacylglycerol, diacylglycerol or triacylglycerol.

The preferred biosynthesis site of fatty acids, oils, lipids or fats in the plants which are advantageously used is, for example, in general the seed or cell strata of the seed, so that seed-specific expression of the nucleic acids used in the process makes sense. However, it is obvious that the biosynthesis of fatty acids, oils or lipids need not be limited to the seed tissue, but can also take place in a tissue-specific manner in all the other parts of the plant, for example in epidermal cells or in the tubers.

If microorganisms such as yeasts, such as Saccharomyces or Schizosaccharomyces, fungi such as Mortierella, Aspergillus, Phytophtora, Entomophthora, Mucor or Thraustochytrium, algae such as Isochrysis, Phaeodactylum or Crypthecodinium are used as organisms in the process according to the invention, these organisms are

advantageously grown in fermentation cultures.

Owing to the use of the nucleic acids according to the invention which code for a lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase and/or lecithin cholesterol acyltransferase, the polyunsaturated fatty acids produced in the process can be increased by at least 5%, preferably by at least 10%, especially preferably by at least 20%, very especially preferably by at least 50% in comparison with the wild type of the organisms which do not comprise the nucleic acids recombinantly.

In principle, the polyunsaturated fatty acids produced by the process according to the invention in the organisms used in the process can be increased in two different ways. Advantageously, the pool of free polyunsaturated fatty acids and/or the content of the esterified polyunsaturated fatty acids produced via the process can be enlarged. Advantageously, the pool of esterified polyunsaturated fatty acids in the transgenic organisms is enlarged by the process according to the invention.

If microorganisms are used as organisms in the process according to the invention, they are grown or cultured in the manner with which the skilled worker is familiar, depending on the host organism. As a rule, microorganisms are grown in a liquid medium comprising a carbon source, usually in the form of sugars, a nitrogen source, usually in the form of organic nitrogen sources such as yeast extract or salts such as ammonium sulfate, trace elements such as salts of iron, manganese and magnesium and, if appropriate, vitamins, at temperatures of between 0°C and 100°C, preferably between 10°C and 60°C, while gassing in oxygen. The pH of the liquid medium can either be kept constant, that is to say regulated during the culturing period, or not. The cultures can be grown batchwise, semibatchwise or continuously. Nutrients can be provided at the beginning of the fermentation or fed in semicontinuously or continuously. The polyunsaturated fatty acids produced can be isolated from the organisms as described above by processes known to the skilled worker, for example by extraction, distillation, crystallization, if appropriate precipitation with salt, and/or chromatography. To this end, the organisms can advantageously be disrupted beforehand.

If the host organisms are microorganisms, the process according to the invention is advantageously carried out at a temperature of between 0°C and 95°C, preferably between 10°C and 85°C, especially preferably between 15°C and 75°C, very especially preferably between 15°C and 45°C.

In this process, the pH value is advantageously kept between pH 4 and 12, preferably between pH 6 and 9, especially preferably between pH 7 and 8.

The process according to the invention can be operated batchwise, semibatchwise or continuously. An overview of known cultivation methods can be found in the textbook by Chmiel (Bioprozeßtechnik 1. Einführung in die Bioverfahrenstechnik [Bioprocess technology 1. Introduction to Bioprocess technology] (Gustav Fischer Verlag, Stuttgart,

1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen [Bioreactors and peripheral equipment] (Vieweg Verlag, Brunswick/Wiesbaden, 1994)).

The culture medium to be used must suitably meet the requirements of the strains in question. Descriptions of culture media for various microorganisms can be found in the textbook "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D.C., USA, 1981).

As described above, these media which can be employed in accordance with the invention usually comprise one or more carbon sources, nitrogen sources, inorganic salts, vitamins and/or trace elements.

Preferred carbon sources are sugars, such as mono-, di- or polysaccharides. Examples of very good carbon sources are glucose, fructose, mannose, galactose, ribose, sorbose, ribulose, lactose, maltose, sucrose, raffinose, starch or cellulose. Sugars can also be added to the media via complex compounds such as molasses or other by-products from sugar refining. The addition of mixtures of a variety of carbon sources may also be advantageous. Other possible carbon sources are oils and fats such as, for example, soya oil, sunflower oil, peanut oil and/or coconut fat, fatty acids such as, for example, palmitic acid, stearic acid and/or linoleic acid, alcohols and/or polyalcohols such as, for example, glycerol, methanol and/or ethanol, and/or organic acids such as, for example, acetic acid and/or lactic acid.

Nitrogen sources are usually organic or inorganic nitrogen compounds or materials comprising these compounds. Examples of nitrogen sources comprise ammonia in liquid or gaseous form or ammonium salts such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate or ammonium nitrate, nitrates, urea, amino acids or complex nitrogen sources such as cornsteep liquor, soya meal, soya protein, yeast extract, meat extract and others. The nitrogen sources can be used individually or as a mixture.

Inorganic salt compounds which may be present in the media comprise the chloride, phosphorus and sulfate salts of calcium, magnesium, sodium, cobalt, molybdenum, potassium, manganese, zinc, copper and iron.

Inorganic sulfur-containing compounds such as, for example, sulfates, sulfites, dithionites, tetrathionates, thiosulfates, sulfides, or else organic sulfur compounds such as mercaptans and thiols may be used as sources of sulfur for the production of sulfur-containing fine chemicals, in particular of methionine.

Phosphoric acid, potassium dihydrogenphosphate or dipotassium hydrogenphosphate or the corresponding sodium-containing salts may be used as sources of phosphorus.

Chelating agents may be added to the medium in order to keep the metal ions in solution. Particularly suitable chelating agents comprise dihydroxyphenols such as catechol or protocatechuate and organic acids such as citric acid.

The fermentation media used according to the invention for culturing microorganisms usually also comprise other growth factors such as vitamins or growth promoters, which include, for example, biotin, riboflavin, thiamine, folic acid, nicotinic acid, panthothenate and pyridoxine. Growth factors and salts are frequently derived from complex media components such as yeast extract, molasses, cornsteep liquor and the like. It is moreover possible to add suitable precursors to the culture medium. The exact composition of the media compounds heavily depends on the particular experiment and is decided upon individually for each specific case. Information on the optimization of media can be found in the textbook "Applied Microbiol. Physiology, A Practical Approach" (Editors P.M. Rhodes, P.F. Stanbury, IRL Press (1997) pp. 53-73, ISBN 0 19 963577 3). Growth media can also be obtained from commercial suppliers, for example Standard 1 (Merck) or BHI (brain heart infusion, DIFCO) and the like.

All media components are sterilized, either by heat (20 min at 1.5 bar and 121°C) or by filter sterilization. The components may be sterilized either together or, if required, separately. All media components may be present at the start of the cultivation or added continuously or batchwise, as desired.

The culture temperature is normally between 15°C and 45°C, preferably at from 25°C to 40°C, and may be kept constant or may be altered during the experiment. The pH of the medium should be in the range from 5 to 8.5, preferably around 7.0. The pH for cultivation can be controlled during cultivation by adding basic compounds such as sodium hydroxide, potassium hydroxide, ammonia and aqueous ammonia or acidic compounds such as phosphoric acid or sulfuric acid. Foaming can be controlled by employing antifoams such as, for example, fatty acid polyglycol esters. To maintain the stability of plasmids it is possible to add to the medium suitable substances having a selective effect, for example antibiotics. Aerobic conditions are maintained by introducing oxygen or oxygen-containing gas mixtures such as, for example, ambient air into the culture. The temperature of the culture is normally 20°C to 45°C and preferably 25°C to 40°C. The culture is continued until formation of the desired product is at a maximum. This aim is normally achieved within 10 to 160 hours.

The fermentation broths obtained in this way, in particular those comprising polyunsaturated fatty acids, usually contain a dry mass of from 7.5 to 25% by weight.

The fermentation broth can then be processed further. The biomass may, according to requirement, be removed completely or partially from the fermentation broth by separation methods such as, for example, centrifugation, filtration, decanting or a combination of these methods or be left completely in said broth. It is advantageous to process the biomass after its separation.

However, the fermentation broth can also be thickened or concentrated without separating the cells, using known methods such as, for example, with the aid of a rotary evaporator, thin-film evaporator, falling-film evaporator, by reverse osmosis or by nanofiltration. Finally, this concentrated fermentation broth can be processed to obtain the fatty acids present therein.

The fatty acids obtained in the process are also suitable as starting material for the chemical synthesis of further products of interest. For example, they can be used in combination with one another or alone for the preparation of pharmaceuticals, foodstuffs, animal feeds or cosmetics.

The invention furthermore relates to isolated nucleic acid sequences coding for polypeptides having lysophosphatidic acid acyltransferase activity, glycerol-3-phosphate acyltransferase activity, diacylglycerol acyltransferase activity or lecithin cholesterol acyltransferase activity, wherein the lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases and/or lecithin cholesterol acyltransferases encoded by the nucleic acid sequences specifically convert C_{18}-, C_{20}-, C_{22}- or C_{24}-fatty acids with at least one double bonds in the fatty acid molecule and advantageously ultimately incorporate these into diacylglycerides and/or triacylglycerides.

Advantageous isolated nucleic acid sequences are sequences selected from the group consisting of:

a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18 or SEQ ID NO: 20,

b) nucleic acid sequences which, as the result of the degeneracy of the genetic code, can be derived from the coding sequence in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18 or SEQ ID NO: 20,

c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18 or SEQ ID NO: 20 which code for polypeptides with the amino acid sequence shown in SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19 or SEQ ID NO: 21 and which have at least 40% homology at the amino acid level with SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19 or SEQ ID NO: 21 and have lysophosphatidic acid acyltransferase activity.

Further advantageous isolated nucleic acid sequences according to the invention are sequences selected from the group consisting of:

a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 22, SEQ ID NO: 24 or SEQ ID NO: 26,

b) nucleic acid sequences which, as the result of the degeneracy of the genetic code, can be derived from the coding sequence in SEQ ID NO: 22, SEQ ID NO: 24 or SEQ ID NO: 26,

c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 22, SEQ ID NO: 24 or SEQ ID NO: 26, which code for polypeptides with the amino acid sequence shown in SEQ ID NO: 23, SEQ ID NO: 25 or SEQ ID NO: 27 and have at least 40% homology at the amino acid level with SEQ ID NO: 23, SEQ ID NO: 25 or SEQ ID NO: 27 and have glycerol-3-phosphate acyltransferase activity.

Additional advantageous isolated nucleic acid sequences according to the invention are sequences selected from the group consisting of:

a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 28, SEQ ID NO: 30 or SEQ ID NO: 32,

b) nucleic acid sequences which, as the result of the degeneracy of the genetic code, can be derived from the coding sequence in SEQ ID NO: 28, SEQ ID NO: 30 or SEQ ID NO: 32,

c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 28, SEQ ID NO: 30 or SEQ ID NO: 32, which code for polypeptides with the amino acid sequence shown in SEQ ID NO: 29, SEQ ID NO: 31 or SEQ ID NO: 33 and have at least 40% homology at the amino acid level with SEQ ID NO: 29, SEQ ID NO: 31 or SEQ ID NO: 33 and which have diacylglycerol acyltransferase activity.

A further group of advantageous isolated nucleic acid sequences according to the invention are sequences selected from the group consisting of:

a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 34 or SEQ ID NO: 36,

b) nucleic acid sequences which, as the result of the degeneracy of the genetic code, can be derived from the coding sequence in SEQ ID NO: 34 or SEQ ID NO: 36,

c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 34 or SEQ ID NO: 36, which code for polypeptides with the amino acid sequence shown in SEQ ID NO: 35 or SEQ ID NO: 37 and which have at least 40% homology at the amino acid level with SEQ ID NO: 35 or SEQ ID NO: 37 and have lecithin cholesterol acyltransferase activity.

With the aid of these isolated nucleic acids according to the invention, LCPUFAs can be incorporated, in LCPUFA-producing organisms, at all positions of, for example, a triacylglycerol, as indicated by the position analyses of the lipids from LCPUFA-producing organisms.

The abovementioned isolated nucleic acid sequences according to the invention can advantageously be combined with the following nucleic acid sequences, which code for polypeptides with acyl-CoA:lysophospholipid acyltransferase activity, selected from the group consisting of:

a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43 or SEQ ID NO: 45,

b) nucleic acid sequences which can be derived from the coding sequence present in SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43 or SEQ ID NO: 45 as the result of the degeneracy of the genetic code,

c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 39, SEQ ID NO: 41, SEQ ID NO: 43 or SEQ ID NO: 45, which code for polypeptides with the amino acid sequence shown in SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44 or SEQ ID NO: 46 and which have at least 40% homology at the amino acid level with SEQ ID NO: 40, SEQ ID NO: 42, SEQ ID NO: 44 or SEQ ID NO: 46 and which have an acyl-CoA:lysophospholipid acyltransferase activity.

All of the nucleic acid sequences used in the process according to the invention are advantageously derived from a eukaryotic organism.

The nucleic acid sequences used in the process which code for proteins with lysophosphatidic acid acyltransferase activity, glycerol-3-phosphate acyltransferase activity, diacylglycerol acyltransferase activity or lecithin cholesterol acyltransferase activity or for proteins of the fatty acid or lipid metabolism, advantageously for proteins with acyl-CoA:lysophospholipid acyltransferase, Δ-4-desaturase, Δ-5-desaturase, Δ-6-desaturase, Δ-8-desaturase, Δ-9-desaturase, Δ-12-desaturase, Δ-5-elongase, Δ-6-elongase or Δ-9-elongase activity are, advantageously alone or preferably in combination, introduced in an expression cassette (= nucleic acid construct) which makes possible the expression of the nucleic acids in an organism, advantageously a plant or a microorganism.

To introduce the nucleic acids used in the process, the latter are advantageously amplified and ligated in the known manner. Preferably, a procedure following the protocol for Pfu DNA polymerase or a Pfu/Taq DNA polymerase mixture is followed. The primers are selected taking into consideration the sequence to be amplified. The primers should expediently be chosen in such a way that the amplificate comprises the entire codogenic sequence from the start codon to the stop codon. After the amplification, the amplificate is expediently analyzed. For example, a gel-electrophoretic separation can be carried out with regards to quality and quantity. Thereafter, the amplificate can be purified following a standard protocol (for example Qiagen). An aliquot of the purified amplificate is then available for the subsequent cloning step. Suitable cloning vectors are generally known to the skilled worker. These include, in particular, vectors which are capable of replication in microbial systems, that is to say mainly vectors which ensure efficient cloning in yeasts or fungi and which make

possible the stable transformation of plants. Those which must be mentioned in particular are various binary and cointegrated vector systems which are suitable for the T-DNA-mediated transformation. Such vector systems are, as a rule, characterized in that they comprise at least the vir genes required for the Agrobacterium-mediated transformation and the T-DNA-delimiting sequences (T-DNA border). These vector systems preferably also comprise further cis-regulatory regions such as promoters and terminators and/or selection markers, by means of which suitably transformed organisms can be identified. While in the case of cointegrated vector systems vir genes and T-DNA sequences are arranged on the same vector, binary systems are based on at least two vectors, one of which bears vir genes, but no T-DNA, while a second one bears T-DNA, but no vir gene. Owing to this fact, the last-mentioned vectors are relatively small, easy to manipulate and to replicate both in E. coli and in Agrobacterium. These binary vectors include vectors from the series pBIB-HYG, pPZP, pBecks, pGreen. In accordance with the invention, Bin19, pBI101, pBinAR, pGPTV and pCAMBIA are used by preference. An overview of binary vectors and their use is found in Hellens et al., Trends in Plant Science (2000) 5, 446–451. In order to prepare the vectors, the vectors can first be linearized with restriction endonuclease(s) and then modified enzymatically in a suitable manner. Thereafter, the vector is purified, and an aliquot is employed for the cloning step. In the cloning step, the enzymatically cleaved and, if appropriate, purified amplificate is cloned using vector fragments which have been prepared in a similar manner, using ligase. In this context, a particular nucleic acid construct, or vector or plasmid construct, can have one or else more than one codogenic gene segment. The codogenic gene segments in these constructs are preferably linked functionally with regulatory sequences. The regulatory sequences include, in particular, plant sequences such as the above-described promoters and terminators. The constructs can advantageously be stably propagated in microorganisms, in particular in Escherichia coli and Agrobacterium tumefaciens, under selective conditions and make possible the transfer of heterologous DNA into plants or microorganisms.

The nucleic acids used in the process, the inventive nucleic acids and nucleic acid constructs, can be introduced into organisms such as microorganisms or advantageously plants, advantageously using cloning vectors, and thus be used in the transformation of plants such as those which are published and cited in: Plant Molecular Biology and Biotechnology (CRC Press, Boca Raton, Florida), Chapter 6/7, pp. 71-119 (1993); F.F. White, Vectors for Gene Transfer in Higher Plants; in: Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press, 1993, 15-38; B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press (1993), 128-143; Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225. Thus, the nucleic acids, the inventive nucleic acids and nucleic acid constructs, and/or vectors used in the process can be used for the recombinant modification of a broad spectrum of organisms, advantageously plants, so that the latter become better and/or more efficient PUFA producers.

A series of mechanisms exists by which the modification of a lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase, or lecithin cholesterol acyltransferase protein according to the invention can influence directly the yield, production and/or production efficiency of a fine chemical from an oil crop plant or a microorganism, owing to a modified protein. The number or activity of the lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase, or lecithin cholesterol acyltransferase protein or gene and also of gene combinations of acyl-CoA:lysophospholipid acyltransferases, desaturases and/or elongases for example may have increased, so that greater amounts of the compounds produced are produced *de novo*, since the organisms lacked this activity and ability to biosynthesize prior to introduction of the corresponding gene(s). This applies analogously to the combination with further desaturases or elongases or further enzymes of the fatty acid and lipid metabolism. The use of various divergent sequences, i.e. sequences which differ at the DNA sequence level, may also be advantageous in this context, or else the use of promoters for gene expression which makes possible a different gene expression in the course of time, for example as a function of the degree of maturity of a seed or an oil-storing tissue.

Owing to the introduction of a lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase, lecithin cholesterol acyltransferase, acyl-CoA:lysophospholipid acyltransferase, desaturase and/or elongase gene or more lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase, lecithin cholesterol acyltransferase, acyl-CoA:lysophospholipid acyltransferase, desaturase and/or elongase genes into an organism, alone or in combination with other genes in a cell, it is not only possible to increase biosynthesis flux towards the end product, but also to increase, or to create *de novo,* the corresponding triacylglycerol composition. Likewise, the number or activity of other genes which are involved in the import of nutrients which are required for the biosynthesis of one or more fine chemicals (e.g. fatty acids, polar and neutral lipids), can be increased, so that the concentration of these precursors, cofactors or intermediates within the cells or within the storage compartment is increased, whereby the ability of the cells to produce PUFAs as described below is enhanced further. Fatty acids and lipids are themselves desirable as fine chemicals; by optimizing the activity or increasing the number of one or more lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase, lecithin cholesterol acyltransferase, acyl-CoA:lysophospholipid acyltransferase, desaturase and/or elongase genes which are involved in the biosynthesis of these compounds, or by destroying the activity of one or more genes which are involved in the degradation of these compounds, an enhanced yield, production and/or efficiency of production of fatty acid and lipid molecules from organisms, advantageously from plants, is made possible.

The isolated nucleic acid molecules used in the process according to the invention code for proteins or parts of these, where the proteins or the individual protein or parts thereof comprise(s) an amino acid sequence with sufficient homology to an amino acid

sequence of the sequence SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35 or SEQ ID NO: 37, so that the protein or part thereof have a and retains an equivalent lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase activity. The protein or part thereof which is encoded by the nucleic acid molecule preferably retains its essential enzymatic activity and the ability to participate in the metabolism of compounds required for the synthesis of cell membranes or lipid bodies in organisms, advantageously in plants, or in the transport of molecules across these membranes. Advantageously, the protein encoded by the nucleic acid molecules is at least approximately 40%, preferably at least approximately 60% and more preferably at least approximately 70%, 80% or 90% and most preferably at least approximately 95%, 96%, 97%, 98%, 99% or more homologous to an amino acid sequence of the sequence SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35 or SEQ ID NO: 37. For the purposes of the invention homology or homologous are to be understood as meaning identity or identical.

Essential enzymatic activity of the inventive lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases or lecithin cholesterol acyltransferases used is understood as meaning that they retain at least an enzymatic activity of at least 10%, preferably 20%, especially preferably 30% and very especially 40% in comparison with the proteins/enzymes encoded by the sequence with SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 and their derivatives and can thus participate in the metabolism of compounds required for the synthesis of fatty acids, fatty acid esters such as diacylglycerides and/or triacylglycerides in an organism, advantageously a plant cell, or in the transport of molecules across membranes, meaning desaturated C_{18}-, C_{20}-, C_{22}- or C_{24}-carbon chains in the fatty acid molecule with double bonds at at least two, advantageously three, four or five positions.

Nucleic acids which can advantageously be used in the process are derived from bacteria, fungi or plants such as algae or mosses, such as the genera Shewanella, Physcomitrella, Thraustochytrium, Fusarium, Phytophtora, Ceratodon, Isochrysis, Aleurita, Muscarioides, Mortierella, Borago, Phaeodactylum, Crypthecodinium or from nematodes such as Caenorhabditis, specifically from the genera and species Shewanella hanedai, Physcomitrella patens, Phytophtora infestans, Fusarium graminaeum, Cryptocodinium cohnii, Ceratodon purpureus, Isochrysis galbana, Aleurita farinosa, Muscarioides viallii, Mortierella alpina, Borago officinalis,

Phaeodactylum tricornutum, or especially advantageously from Caenorhabditis elegans.

Alternatively, the isolated nucleotide sequences used may code for lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases or lecithin cholesterol acyltransferases which hybridize with a nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36, for example under stringent conditions.

The nucleic acid sequences used in the process are advantageously introduced into an expression cassette which makes possible the expression of the nucleic acids in organisms such as microorganisms or plants.

In doing so, the nucleic acid sequences which code for the lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases or lecithin cholesterol acyltransferases of the invention, and the nucleic acid sequences which code for the acyl-CoA:lysophospholipid acyltransferases used in combination, the desaturases and/or the elongases are linked functionally with one or more regulatory signals, advantageously for enhancing gene expression. These regulatory sequences are intended to make possible the specific expression of the genes and proteins. Depending on the host organism, this may mean, for example, that the gene is expressed and/or overexpressed only after induction has taken place, or else that it expresses and/or overexpresses immediately. For example, these regulatory sequences take the form of sequences to which inductors or repressors bind, thus controlling the expression of the nucleic acid. In addition to these novel regulatory sequences, or instead of these sequences, the natural regulation of these sequences may still be present before the actual structural genes and, if appropriate, may have been genetically modified in such a way that natural regulation has been eliminated and expression of the genes has been enhanced. However, the expression cassette (= expression construct = gene construct) can also be simpler in construction, that is to say no additional regulatory signals have been inserted before the nucleic acid sequence or its derivatives, and the natural promoter together with its regulation has not been removed. Instead, the natural regulatory sequence has been mutated in such a way that regulation no longer takes place and/or gene expression is enhanced. These modified promoters can also be positioned on their own before the natural gene in the form of part-sequences (= promoter with parts of the nucleic acid sequences of the invention) in order to enhance the activity. Moreover, the gene construct may advantageously also comprise one or more of what are known as enhancer sequences in functional linkage with the promoter, which make possible an enhanced expression of the nucleic acid sequence. Additional advantageous sequences, such as further regulatory elements or terminators, may also be inserted at the 3' end of the DNA sequences. The lysophosphatidic acid acyltransferase, glycerol-3-phosphate

acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase genes and the advantageously used acyl-CoA:lysophospholipid acyltransferase, Δ-4-desaturase, Δ5-desaturase, Δ-6-desaturase and/or Δ-8-desaturase genes and/or Δ-5-elongase, Δ-6-elongase and/or Δ-9-elongase genes may be present in one or more copies in the expression cassette (= gene construct). Preferably, only one copy of the genes is present in each expression cassette. This gene construct or the gene constructs can be expressed together in the host organism. In this context, the gene construct(s) can be inserted in one or more vectors and be present in the cell in free form, or else be inserted in the genome. It is advantageous for the insertion of further genes in the host genome when the genes to be expressed are present together in one gene construct.

In this context, the regulatory sequences or factors can, as described above, preferably have a positive effect on the gene expression of the genes introduced, thus enhancing it. Thus, an enhancement of the regulatory elements, advantageously at the transcriptional level, may take place by using strong transcription signals such as promoters and/or enhancers. In addition, however, enhanced translation is also possible, for example by improving the stability of the mRNA.

A further embodiment of the invention is one or more gene constructs which comprise one or more sequences which are defined by SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 or its derivatives and which code for polypeptides as shown in SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35 or SEQ ID NO: 37. The abovementioned lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases or lecithin cholesterol acyltransferases lead advantageously to an exchange or incorporation of fatty acids between the mono-, di- and/or triglyceride pool of the cell and the CoA-fatty acid ester pool, the substrate advantageously having one, two, three, four or five double bonds and advantageously 18, 20, 22 or 24 carbon atoms in the fatty acid molecule. The same applies to their homologs, derivatives or analogs, which are linked functionally with one or more regulatory signals, advantageously for enhancing gene expression.

Advantageous regulatory sequences for the novel process are present for example in promoters such as the cos, tac, trp, tet, trp-tet, lpp, lac, lpp-lac, laclq, T7, T5, T3, gal, trc, ara, SP6, λ-PR or λ-PL promoter and are advantageously employed in Gram-negative bacteria. Further advantageous regulatory sequences are, for example, present in the Gram-positive promoters amy and SPO2, in the yeast or fungal promoters ADC1, MFα, AC, P-60, CYC1, GAPDH, TEF, rp28, ADH or in the plant promoters CaMV/35S [Franck et al., Cell 21 (1980) 285–294], PRP1 [Ward et al., Plant.

Mol. Biol. 22 (1993)], SSU, OCS, lib4, usp, STLS1, B33, nos or in the ubiquitin or phaseolin promoter. Advantageous in this context are also inducible promoters, such as the promoters described in EP-A-0 388 186 (benzylsulfonamide-inducible), Plant J. 2, 1992:397–404 (Gatz et al., tetracycline-inducible), EP–A–0 335 528 (abscisic acid-inducible) or WO 93/21334 (ethanol- or cyclohexenol-inducible). Further suitable plant promoters are the cytosolic FBPase promoter or the ST-LSI promoter of potato (Stockhaus et al., EMBO J. 8, 1989, 2445), the glycine max phosphoribosyl-pyrophosphate amidotransferase promoter (Genbank Accession No. U87999) or the node-specific promoter described in EP–A–0 249 676. Especially advantageous promoters are promoters which make possible the expression in tissues which are involved in the biosynthesis of fatty acids. Very especially advantageous are seed-specific promoters, such as the USP promoter as described, but also other promoters such as the LeB4, DC3, phaseolin or napin promoter. Further especially advantageous promoters are seed-specific promoters which can be used for monocotyledonous or dicotyledonous plants and which are described in US 5,608,152 (oilseed rape napin promoter), WO 98/45461 (Arabidopsis oleosin promoter), US 5,504,200 (Phaseolus vulgaris phaseolin promoter), WO 91/13980 (Brassica Bce4 promoter), by Baeumlein et al., Plant J., 2, 2, 1992:233–239 (LeB4 promoter from a legume), these promoters being suitable for dicots. Examples of promoters which are suitable for monocots are the barley lpt–2 or lpt–1 promoter (WO 95/15389 and WO 95/23230), the barley hordein promoter and other suitable promoters described in WO 99/16890.

In principle, it is possible to use all natural promoters together with their regulatory sequences, such as those mentioned above, for the novel process. It is also possible and advantageous to use synthetic promoters, either in addition or alone, in particular when they mediate seed-specific expression, such as those described in WO 99/16890.

In order to achieve a particularly high PUFA content, especially in transgenic plants, the PUFA biosynthesis genes should advantageously be expressed in oil crops in a seed-specific manner. To this end, seed-specific promoters can be used, or those promoters which are active in the embryo and/or in the endosperm. In principle, seed-specific promoters can be isolated both from dicotyledonous and from monocotyledonous plants. Advantageous preferred promoters are listed hereinbelow: USP (= unknown seed protein) and vicilin (Vicia faba) [Bäumlein et al., Mol. Gen Genet., 1991, 225(3)], napin (oilseed rape) [US 5,608,152], acyl carrier protein (oilseed rape) [US 5,315,001 and WO 92/18634], oleosin (Arabidopsis thaliana) [WO 98/45461 and WO 93/20216], phaseolin (Phaseolus vulgaris) [US 5,504,200], Bce4 [WO 91/13980], legumes B4 (LegB4 promoter) [Bäumlein et al., Plant J., 2,2, 1992], Lpt2 and lpt1 (barley) [WO 95/15389 and WO 95/23230], seed-specific promoters from rice, maize and wheat [WO 99/16890], Amy32b, Amy 6-6 and aleurain [US 5,677,474], Bce4 (oilseed rape) [US 5,530,149], glycinin (soybean) [EP 571 741], phosphoenol pyruvate carboxylase (soybean) [JP 06/62870], ADR12-2 (soybean) [WO 98/08962], isocitrate lyase (oilseed rape) [US 5,689,040] or α-amylase (barley) [EP 781 849].

Plant gene expression can also be facilitated via a chemically inducible promoter (see review in Gatz 1997, Annu. Rev. Plant Physiol. Plant Mol. Biol., 48:89-108). Chemically inducible promoters are particularly suitable when it is desired that gene expression should take place in a time-specific manner. Examples of such promoters are a salicylic-acid-inducible promoter (WO 95/19443), a tetracycline-inducible promoter (Gatz et al. (1992) Plant J. 2, 397-404) and an ethanol-inducible promoter.

To ensure the stable integration of the biosynthesis genes into the transgenic plant over a plurality of generations, each of the nucleic acids which code for lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase and/or lecithin cholesterol acyltransferase, the advantageous acyl-CoA:lysophospholipid acyltransferase, Δ-4-desaturase, Δ-5-desaturase, Δ-6-desaturase, Δ-8-desaturase and/or Δ-5-elongase, Δ-6-elongase and/or Δ-9-elongase and which are used in the process should be expressed under the control of a separate promoter, preferably a promoter which differs from the other promoters, since repeating sequence motifs can lead to instability of the T-DNA, or to recombination events. In this context, the expression cassette is advantageously constructed in such a way that a promoter is followed by a suitable cleavage site, advantageously in a polylinker, for insertion of the nucleic acid to be expressed and, if appropriate, a terminator is positioned behind the polylinker. This sequence is repeated several times, preferably three, four or five times, so that up to five genes can be combined in one construct and introduced into the transgenic plant in order to be expressed. Advantageously, the sequence is repeated up to three times. To express the nucleic acid sequences, the latter are inserted behind the promoter via the suitable cleavage site, for example in the polylinker. Advantageously, each nucleic acid sequence has its own promoter and, if appropriate, its own terminator. However, it is also possible to insert a plurality of nucleic acid sequences behind a promoter and, if appropriate, before a terminator. Here, the insertion site, or the sequence, of the inserted nucleic acids in the expression cassette is not of critical importance, that is to say a nucleic acid sequence can be inserted at the first or last position in the cassette without its expression being substantially influenced thereby. Advantageously, different promoters such as, for example, the USP, LegB4 or DC3 promoter, and different terminators can be used in the expression cassette. However, it is also possible to use only one type of promoter in the cassette. This, however, may lead to undesired recombination events.

As described above, the transcription of the genes which have been introduced should advantageously be terminated by suitable terminators at the 3' end of the biosynthesis genes which have been introduced (behind the stop codon). An example of a sequence which can be used in this context is the OCS1 terminator. As is the case with the promoters, different terminator sequences should be used for each gene.

As described above, the gene construct can also comprise further genes to be introduced into the organisms. It is possible and advantageous to introduce into the host organisms, and to express therein, regulatory genes such as genes for inductors,

repressors or enzymes which, owing to their enzyme activity, engage in the regulation of one or more genes of a biosynthetic pathway. These genes can be of heterologous or of homologous origin. Moreover, further biosynthesis genes of the fatty acid or lipid metabolism can advantageously be present in the nucleic acid construct, or gene construct; however, these genes can also be positioned on one or more further nucleic acid constructs. Biosynthesis genes of the fatty acid or lipid metabolism which are advantageously used are a gene selected from the group consisting of acyl-CoA dehydrogenase(s), acyl-ACP [= acyl carrier protein] desaturase(s), acyl-ACP thioesterase(s), fatty acid acyltransferase(s), acyl-CoA:lysophospholipid acyltransferase(s), fatty acid synthase(s), fatty acid hydroxylase(s), acetyl-coenzyme A carboxylase(s), acyl-coenzyme A oxidase(s), fatty acid desaturase(s), fatty acid acetylenases, lipoxygenase(s), triacylglycerol lipase(s), alleneoxide synthase(s), hydroperoxide lyase(s) or fatty acid elongase(s) or combinations thereof. Especially advantageous nucleic acid sequences are biosynthesis genes of the fatty acid or lipid metabolism selected from the group consisting of acyl-CoA:lysophospholipid acyltransferase, Δ-4-desaturase, Δ-5-desaturase, Δ-6-desaturase, Δ-8-desaturase, Δ-9-desaturase, Δ-12-desaturase, Δ-5-elongase, Δ-6-elongase or Δ-9-elongase.

In this context, the abovementioned nucleic acids and genes can be cloned into expression cassettes of the invention in combination with other elongases and desaturases and used for transforming plants with the aid of Agrobacterium.

Here, the regulatory sequences or factors can, as described above, preferably have a positive effect on, and thus enhance, the expression of the genes which have been introduced. Thus, enhancement of the regulatory elements can advantageously take place at the transcriptional level by using strong transcription signals such as promoters and/or enhancers. However, an enhanced translation is also possible, for example by improving the stability of the mRNA. In principle, the expression cassettes can be used directly for introduction into the plant or else be introduced into a vector.

These advantageous vectors, preferably expression vectors, comprise the nucleic acids which code for lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases or lecithin cholesterol acyltransferases and which are used in the process, or else a nucleic acid construct which comprises the nucleic acid used either alone or in combination with further biosynthesis genes of the fatty acid or lipid metabolism such as the acyl-CoA:lysophospholipid acyltransferases, Δ-4-desaturase, Δ-5-desaturase, Δ-6-desaturase, Δ-8-desaturase, Δ-9-desaturase, Δ-12-desaturase, Δ-5-elongase, Δ-6-elongase and/or Δ-9-elongase. As used in the present context, the term "vector" refers to a nucleic acid molecule which is capable of transporting another nucleic acid to which it is bound. One type of vector is a "plasmid", a circular double-stranded DNA loop into which additional DNA segments can be ligated. A further type of vector is a viral vector, it being possible for additional DNA segments to be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they have been introduced (for example bacterial vectors with bacterial replication origin). Other vectors are

advantageously integrated into the genome of a host cell when they are introduced into the host cell, and thus replicate together with the host genome. Moreover, certain vectors can govern the expression of genes with which they are in functional linkage. These vectors are referred to in the present context as "expression vectors". Usually, expression vectors which are suitable for DNA recombination techniques take the form of plasmids. In the present description, "plasmid" and "vector" can be used exchangeably since the plasmid is the form of vector which is most frequently used. However, the invention is intended to comprise these other forms of expression vectors, such as viral vectors, which exert similar functions. Furthermore, the term "vector" is also intended to comprise other vectors with which the skilled worker is familiar, such as phages, viruses such as SV40, CMV, TMV, transposons, IS elements, phasmids, phagemids, cosmids, linear or circular DNA.

The recombinant expression vectors advantageously used in the process comprise the nucleic acids described below or the above-described gene construct in a form which is suitable for expressing the nucleic acids used in a host cell, which means that the recombinant expression vectors comprise one or more regulatory sequences, selected on the basis of the host cells to be used for the expression, which regulatory sequence(s) is/are linked functionally with the nucleic acid sequence to be expressed. In a recombinant expression vector, "linked functionally" means that the nucleotide sequence of interest is bound to the regulatory sequence(s) in such a way that the expression of the nucleotide sequence is possible and they are bound to each other in such a way that both sequences carry out the predicted function which is ascribed to the sequence (for example in an in-vitro transcription/translation system, or in a host cell if the vector is introduced into the host cell). The term "regulatory sequence" is intended to comprise promoters, enhancers and other expression control elements (for example polyadenylation signals). These regulatory sequences are described, for example, in Goeddel: Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990), or see: Gruber and Crosby, in: Methods in Plant Molecular Biology and Biotechnology, CRC Press, Boca Raton, Florida, Ed.: Glick and Thompson, Chapter 7, 89-108, including the references cited therein. Regulatory sequences comprise those which govern the constitutive expression of a nucleotide sequence in many types of host cell and those which govern the direct expression of the nucleotide sequence only in specific host cells under specific conditions. The skilled worker knows that the design of the expression vector can depend on factors such as the choice of host cell to be transformed, the expression level of the desired protein and the like.

The recombinant expression vectors used can be designed for the expression of lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases or lecithin cholesterol acyltransferases, acyl-CoA:lysophospholipid acyltransferases, desaturases and elongases in prokaryotic or eukaryotic cells. This is advantageous since intermediate steps of the vector construction are frequently carried out in microorganisms for the sake of simplicity. For example, lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase,

diacylglycerol acyltransferase, lecithin cholesterol acyltransferase, acyl-CoA:lysophospholipid acyltransferase, desaturase and/or elongase genes can be expressed in bacterial cells, insect cells (using Baculovirus expression vectors), yeast and other fungal cells (see Romanos, M.A., et al. (1992) "Foreign gene expression in yeast: a review", Yeast 8:423-488; van den Hondel, C.A.M.J.J., et al. (1991) "Heterologous gene expression in filamentous fungi", in: More Gene Manipulations in Fungi, J.W. Bennet & L.L. Lasure, Ed., pp. 396-428: Academic Press: San Diego; and van den Hondel, C.A.M.J.J., & Punt, P.J. (1991) "Gene transfer systems and vector development for filamentous fungi, in: Applied Molecular Genetics of Fungi, Peberdy, J.F., et al., Ed., pp. 1-28, Cambridge University Press: Cambridge), algae (Falciatore et al., 1999, Marine Biotechnology.1, 3:239-251), ciliates of the types: Holotrichia, Peritrichia, Spirotrichia, Suctoria, Tetrahymena, Paramecium, Colpidium, Glaucoma, Platyophrya, Potomacus, Desaturaseudocohnilembus, Euplotes, Engelmaniella and Stylonychia, in particular of the genus Stylonychia lemnae, using vectors in a transformation method as described in WO 98/01572 and, preferably, in cells of multi-celled plants (see Schmidt, R. and Willmitzer, L. (1988) "High efficiency Agrobacterium tumefaciens-mediated transformation of Arabidopsis thaliana leaf and cotyledon explants" Plant Cell Rep.: 583-586; Plant Molecular Biology and Biotechnology, C Press, Boca Raton, Florida, Chapter 6/7, pp. 71-119 (1993); F.F. White, B. Jenes et al., Techniques for Gene Transfer, in: Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press (1993), 128-43; Potrykus, Annu. Rev. Plant Physiol. Plant Molec. Biol. 42 (1991), 205-225 (and references cited therein)). Suitable host cells are furthermore discussed in Goeddel, Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, CA (1990). As an alternative, the recombinant expression vector can be transcribed and translated in vitro, for example using T7-promoter regulatory sequences and T7-polymerase.

In most cases, the expression of proteins in prokaryotes involves the use of vectors comprising constitutive or inducible promoters which govern the expression of fusion or nonfusion proteins. Typical fusion expression vectors are, inter alia, pGEX (Pharmacia Biotech Inc; Smith, D.B., and Johnson, K.S. (1988) Gene 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ), where glutathione S-transferase (GST), maltose-E binding protein and protein A, respectively, is fused with the recombinant target protein.

Examples of suitable inducible nonfusion E. coli expression vectors are, inter alia, pTrc (Amann et al. (1988) Gene 69:301-315) and pET 11d (Studier et al., Gene Expression Technology: Methods in Enzymology 185, Academic Press, San Diego, California (1990) 60-89). The target gene expression from the pTrc vector is based on the transcription from a hybrid trp-lac fusion promoter by the host RNA polymerase. The target gene expression from the vector pET 11d is based on the transcription of a T7-gn10-lac fusion promoter, which is mediated by a viral RNA polymerase (T7 gn1), which is coexpressed. This viral polymerase is provided by the host strains BL21 (DE3) or HMS174 (DE3) from a resident λ-prophage which harbors a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

Other vectors which are suitable for prokaryotic organisms are known to the skilled worker, these vectors are, for example in E. coli pLG338, pACYC184, the pBR series such as pBR322, the pUC series such as pUC18 or pUC19, the M113mp series, pKC30, pRep4, pHS1, pHS2, pPLc236, pMBL24, pLG200, pUR290, pIN-III113-B1, λgt11 or pBdCI, in Streptomyces pIJ101, pIJ364, pIJ702 or pIJ361, in Bacillus pUB110, pC194 or pBD214, in Corynebacterium pSA77 or pAJ667.

In a further embodiment, the expression vector is a yeast expression vector. Examples for vectors for expression in the yeast S. cerevisiae comprise pYeDesaturasec1 (Baldari et al. (1987) Embo J. 6:229-234), pMFa (Kurjan and Herskowitz (1982) Cell 30:933-943), pJRY88 (Schultz et al. (1987) Gene 54:113-123) and pYES2 (Invitrogen Corporation, San Diego, CA). Vectors and processes for the construction of vectors which are suitable for use in other fungi, such as the filamentous fungi, comprise those which are described in detail in: van den Hondel, C.A.M.J.J., & Punt, P.J. (1991) "Gene transfer systems and vector development for filamentous fungi, in: Applied Molecular Genetics of fungi, J.F. Peberdy et al., Ed., pp. 1-28, Cambridge University Press: Cambridge, or in: More Gene Manipulations in Fungi [J.W. Bennet & L.L. Lasure, Ed., pp. 396-428: Academic Press: San Diego]. Further suitable yeast vectors are, for example, pAG-1, YEp6, YEp13 or pEMBLYe23.

As an alternative, the lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases, lecithin cholesterol acyltransferases, acyl-CoA:lysophospholipid acyltransferases, desaturases and/or elongases can be expressed in insect cells using Baculovirus expression vectors. Baculovirus vectors which are available for the expression of proteins in cultured insect cells (for example Sf9 cells) comprise the pAc series (Smith et al. (1983) Mol. Cell Biol. 3:2156-2165) and the pVL series (Lucklow and Summers (1989) Virology 170:31-39).

The abovementioned vectors offer only a small overview of suitable vectors which are possible. Further plasmids are known to the skilled worker and are described, for example, in: Cloning Vectors (Ed. Pouwels, P.H., et al., Elsevier, Amsterdam-New York-Oxford, 1985, ISBN 0 444 904018). For further suitable expression systems for prokaryotic and eukaryotic cells, see the Chapters 16 and 17 in Sambrook, J., Fritsch, E.F., and Maniatis, T., Molecular Cloning: A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In a further embodiment of the process, the lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases, lecithin cholesterol acyltransferases, acyl-CoA:lysophospholipid acyltransferases, desaturases and/or elongases can be expressed in single-celled plant cells (such as algae), see Falciatore et al., 1999, Marine Biotechnology 1 (3):239-251 and references cited therein, and in plant cells from higher plants (for example spermatophytes such as arable crops). Examples of plant expression vectors comprise those which are described in detail in: Becker, D., Kemper, E., Schell, J., and Masterson, R. (1992)

"New plant binary vectors with selectable markers located proximal to the left border", Plant Mol. Biol. 20:1195-1197; and Bevan, M.W. (1984) "Binary Agrobacterium vectors for plant transformation", Nucl. Acids Res. 12:8711-8721; Vectors for Gene Transfer in Higher Plants; in: Transgenic Plants, Vol. 1, Engineering and Utilization, Ed.: Kung and R. Wu, Academic Press, 1993, pp. 15-38.

A plant expression cassette preferably comprises regulatory sequences which are capable of governing the expression of genes in plant cells and which are linked functionally so that each sequence can fulfill its function, such as transcriptional termination, for example polyadenylation signals. Preferred polyadenylation signals are those which are derived from Agrobacterium tumefaciens T-DNA, such as gene 3 of the Ti plasmid pTiACH5 (Gielen et al., EMBO J. 3 (1984) 835 et seq.), which is known as octopine synthase, or functional equivalents thereof, but all other terminators which are functionally active in plants are also suitable.

Since plant gene expression is very often not limited to transcriptional levels, a plant expression cassette preferably comprises other sequences which are linked functionally, such as translation enhancers, for example the overdrive sequence, which comprises the tobacco mosaic virus 5'–untranslated leader sequence, which increases the protein/RNA ratio (Gallie et al., 1987, Nucl. Acids Research 15:8693-8711).

As described above, plant gene expression must be linked functionally with a suitable promoter which triggers gene expression with the correct timing or in a cell- or tissue-specific manner. Utilizable promoters are constitutive promoters (Benfey et al., EMBO J. 8 (1989) 2195-2202), such as those which are derived from plant viruses, such as 35S CAMV (Franck et al., Cell 21 (1980) 285-294), 19S CaMV (see also US 5352605 and WO 84/02913), or plant promoters, such as the promoter of the small rubisco subunit, which is described in US 4,962,028.

Other preferred sequences for use in functional linkage in plant gene expression cassettes are targeting sequences, which are required for steering the gene product into its corresponding cell compartment (see a review in Kermode, Crit. Rev. Plant Sci. 15, 4 (1996) 285-423 and references cited therein), for example into the vacuole, into the nucleus, all types of plastids, such as amyloplasts, chloroplasts, chromoplasts, the extracellular space, the mitochondria, the endoplasmic reticulum, elaioplasts, peroxisomes and other compartments of plant cells.

As described above, plant gene expression can also be facilitated via a chemically inducible promoter (see review in Gatz 1997, Annu. Rev. Plant Physiol. Plant Mol. Biol., 48:89-108). Chemically inducible promoters are particularly suitable when it is desired that the gene expression takes place in a time-specific manner. Examples of such promoters are a salicylic-acid-inducible promoter (WO 95/19443), a tetracyclin-inducible promoter (Gatz et al. (1992) Plant J. 2, 397-404) and an ethanol-inducible promoter.

Promoters which respond to biotic or abiotic stress conditions are also suitable, for example the pathogen-induced PRP1 gene promoter (Ward et al., Plant. Mol. Biol. 22

(1993) 361-366), the heat-inducible tomato hsp80 promoter (US 5,187,267), the chill-inducible potato alpha-amylase promoter (WO 96/12814) or the wound-inducible pinII promoter (EP-A-0 375 091).

Especially preferred are those promoters which bring about the gene expression in tissues and organs in which the biosynthesis of fatty acids, lipids and oils takes place, in seed cells, such as cells of the endosperm and of the developing embryo. Suitable promoters are the oilseed rape napin gene promoter (US 5,608,152), the Vicia faba USP promoter (Baeumlein et al., Mol Gen Genet, 1991, 225 (3):459-67), the Arabidopsis oleosin promoter (WO 98/45461), the Phaseolus vulgaris phaseolin promoter (US 5,504,200), the Brassica Bce4 promoter (WO 91/13980) or the legumine B4 promoter (LeB4; Baeumlein et al., 1992, Plant Journal, 2 (2):233-9), and promoters which bring about the seed-specific expression in monocotyledonous plants such as maize, barley, wheat, rye, rice and the like. Suitable noteworthy promoters are the barley lpt2 or lpt1 gene promoter (WO 95/15389 and WO 95/23230) or the promoters from the barley hordein gene, the rice glutelin gene, the rice oryzin gene, the rice prolamine gene, the wheat gliadine gene, the wheat glutelin gene, the maize zeine gene, the oat glutelin gene, the sorghum kasirin gene or the rye secalin gene, which are described in WO 99/16890.

In particular, it may be desired to bring about the multiparallel expression of the lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases or lecithin cholesterol acyltransferases used in the process alone or in combination with acyl-CoA:lysophospholipid acyltransferases, desaturases and/or elongases. Such expression cassettes can be introduced via the simultaneous transformation of a plurality of individual expression constructs or, preferably, by combining a plurality of expression cassettes on one construct. Also, a plurality of vectors can be transformed with in each case a plurality of expression cassettes and then transferred onto the host cell.

Promoters which are likewise especially suitable are those which bring about plastid-specific expression, since plastids constitute the compartment in which the precursors and some end products of lipid biosynthesis are synthesized. Suitable promoters, such as the viral RNA polymerase promoter, are described in WO 95/16783 and WO 97/06250, and the clpP promoter from Arabidopsis, described in WO 99/46394.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. The terms "transformation" and "transfection", conjugation and transduction, as used in the present context, are intended to comprise a multiplicity of methods known in the prior art for the introduction of foreign nucleic acid (for example DNA) into a host cell, including calcium phosphate or calcium chloride coprecipitation, DEAE-dextran-mediated transfection, lipofection, natural competence, chemically mediated transfer, electroporation or particle bombardment. Suitable methods for the transformation or transfection of host cells, including plant cells, can be found in Sambrook et al. (Molecular Cloning: A Laboratory

Manual., 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) and other laboratory textbooks such as Methods in Molecular Biology, 1995, Vol. 44, Agrobacterium protocols, Ed.: Gartland and Davey, Humana Press, Totowa, New Jersey.

Host cells which are suitable in principle for taking up the nucleic acid according to the invention, the gene product according to the invention or the vector according to the invention are all prokaryotic or eukaryotic organisms. The host organisms which are advantageously used are microorganisms such as fungi or yeasts, or plant cells, preferably plants or parts thereof. Fungi, yeasts or plants are preferably used, especially preferably plants, very especially preferably plants such as oil crop plants, which are high in lipid compounds, such as oilseed rape, evening primrose, hemp, thistle, peanut, canola, linseed, soybean, safflower, sunflower, borage, or plants such as maize, wheat, rye, oats, triticale, rice, barley, cotton, cassava, pepper, Tagetes, Solanaceae plants such as potato, tobacco, eggplant and tomato, Vicia species, pea, alfalfa, bushy plants (coffee, cacao, tea), Salix species, trees (oil palm, coconut), and perennial grasses and fodder crops. Especially preferred plants according to the invention are oil crop plants such as soybean, peanut, oilseed rape, canola, linseed, hemp, evening primrose, sunflower, safflower, trees (oil palm, coconut).

The invention furthermore relates to isolated nucleic acid sequences as described above coding for polypeptides having lysophosphatidic acid acyltransferase activity, glycerol-3-phosphate acyltransferase activity, diacylglycerol acyltransferase activity or lecithin cholesterol acyltransferase activity, where the lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases or lecithin cholesterol acyltransferases encoded by the nucleic acid sequences specifically convert C_{18}-, C_{20}-, C_{22}- or C_{24}-fatty acids with at least one double bonds in the fatty acid molecule.

Advantageous isolated nucleic acid sequences are sequences selected from the group consisting of:

a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18 or SEQ ID NO: 20,

b) nucleic acid sequences which, as the result of the degeneracy of the genetic code, can be derived from the coding sequence in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18 or SEQ ID NO: 20,

c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18 or SEQ ID

NO: 20, which code for polypeptides with the amino acid sequence shown in SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19 or SEQ ID NO: 21 and which have at least 40% homology at the amino acid level with SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19 or SEQ ID NO: 21 and have lysophosphatidic acid acyltransferase activity.

Further advantageous isolated nucleic acid sequences are sequences selected from the group consisting of:

a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 22, SEQ ID NO: 24 or SEQ ID NO: 26,

b) nucleic acid sequences which, as the result of the degeneracy of the genetic code, can be derived from the coding sequence in SEQ ID NO: 22, SEQ ID NO: 24 or SEQ ID NO: 26,

c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 22, SEQ ID NO: 24 or SEQ ID NO: 26, which code for polypeptides with the amino acid sequence shown in SEQ ID NO: 23, SEQ ID NO: 25 or SEQ ID NO: 27 and have at least 40% homology at the amino acid level with SEQ ID NO: 23, SEQ ID NO: 25 or SEQ ID NO: 27 and have glycerol-3-phosphate acyltransferase activity.

Further advantageous isolated nucleic acid sequences are sequences selected from the group consisting of:

a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 28, SEQ ID NO: 30 or SEQ ID NO: 32,

b) nucleic acid sequences which, as the result of the degeneracy of the genetic code, can be derived from the coding sequence in SEQ ID NO: 28, SEQ ID NO: 30 or SEQ ID NO: 32,

c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 28, SEQ ID NO: 30 or SEQ ID NO: 32, which code for polypeptides with the amino acid sequence shown in SEQ ID NO: 29, SEQ ID NO: 31 or SEQ ID NO: 33 and have at least 40% homology at the amino acid level with SEQ ID NO: 29, SEQ ID NO: 31 or SEQ ID NO: 33 and which have diacylglycerol acyltransferase activity.

Further advantageous isolated nucleic acid sequences are sequences selected from the group consisting of:

a) a nucleic acid sequence with the sequence shown in SEQ ID NO: 34 or SEQ ID NO: 36,

b) nucleic acid sequences which, as the result of the degeneracy of the genetic code, can be derived from the coding sequence in SEQ ID NO: 34 or SEQ ID NO: 36,

c) derivatives of the nucleic acid sequence shown in SEQ ID NO: 34 or SEQ ID NO: 36, which code for polypeptides with the amino acid sequence shown in SEQ ID NO: 35 or SEQ ID NO: 37 and which have at least 40% homology at the amino acid level with SEQ ID NO: 35 or SEQ ID NO: 37 and have lecithin cholesterol acyltransferase activity.

The abovementioned nucleic acids according to the invention are derived from organisms such as animals, ciliates, fungi, plants such as algae or dinoflagellates which are capable of synthesizing PUFAs.

In an advantageous embodiment, the term "nucleic acid (molecule)" as used in the present context additionally comprises the untranslated sequence at the 3' and at the 5' end of the coding gene region: at least 500, preferably 200, especially preferably 100 nucleotides of the sequence upstream of the 5' end of the coding region and at least 100, preferably 50, especially preferably 20 nucleotides of the sequence downstream of the 3' end of the coding gene region. An "isolated" nucleic acid molecule is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. An "isolated" nucleic acid preferably has no sequences which naturally flank the nucleic acid in the genomic DNA of the organism from which the nucleic acid is derived (for example sequences which are located at the 5' and 3' ends of the nucleic acid). In various embodiments, the isolated lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase and/or lecithin cholesterol acyltransferase molecule can comprise for example fewer than approximately 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in the genomic DNA of the cell from which the nucleic acid is derived.

The nucleic acid molecules used in the process, for example a nucleic acid molecule with a nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 or of a part thereof can be isolated using molecular-biological standard techniques and the sequence information provided herein. Also, for example a homologous sequence or homologous, conserved sequence regions can be identified at the DNA or amino acid level with the aid of comparative algorithms. They can be used as hybridization probe together with standard hybridization techniques (such as, for example, those described in Sambrook et al., Molecular Cloning: A Laboratory Manual. 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989) for isolating further nucleic acid sequences which can be used in the process. Moreover, a nucleic acid molecule

comprising a complete sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 or a part thereof can be isolated by polymerase chain reaction, where oligonucleotide primers which are based on this sequence or on parts thereof are used (for example a nucleic acid molecule comprising the complete sequence or a part thereof can be isolated by polymerase chain reaction using oligonucleotide primers which have been generated based on this same sequence). For example, mRNA can be isolated from cells (for example by means of the guanidinium thiocyanate extraction method of Chirgwin et al. (1979) Biochemistry 18:5294-5299) and cDNA by means of reverse transcriptase (for example Moloney MLV reverse transcriptase, available from Gibco/BRL, Bethesda, MD, or AMV reverse transcriptase, available from Seikagaku America, Inc., St. Petersburg, FL). Synthetic oligonucleotide primers for the amplification by means of polymerase chain reaction can be generated based on one of the sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 or with the aid of the amino acid sequences detailed in SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35 or SEQ ID NO: 37. A nucleic acid according to the invention can be amplified by standard PCR amplification techniques using cDNA or, alternatively, genomic DNA as template and suitable oligonucleotide primers. The nucleic acid amplified thus can be cloned into a suitable vector and characterized by means of DNA sequence analysis. Oligonucleotides which correspond to a desaturase nucleotide sequence can be generated by standard synthetic methods, for example using an automatic DNA synthesizer.

Homologs of the lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase nucleic acid sequences used with the sequence SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 means, for example, allelic variants with at least approximately 40 to 60%, preferably at least approximately from 60 to 70%, more preferably at least approximately from 70 to 80%, 80 to 90% or 90 to 95% and even more preferably at least approximately 95%, 96%, 97%, 98%, 99% or more homology with a nucleotide sequence shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID

NO: 34 or SEQ ID NO: 36 or its homologs, derivatives or analogs or parts thereof. Furthermore, isolated nucleic acid molecules of a nucleotide sequence which hybridize with one of the nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 or with a part thereof, for example hybridized under stringent conditions. Allelic variants comprise in particular functional variants which can be obtained by deletion, insertion or substitution of nucleotides from/into the sequence detailed in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO:6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 it being intended, however, that the enzyme activity of the resulting proteins which are synthesized is advantageously retained for the insertion of one or more genes. Proteins which retain the enzymatic activity of lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase, i.e. whose activity is essentially not reduced, means proteins with at least 10%, preferably 20%, especially preferably 30%, very especially preferably 40% of the original enzyme activity in comparison with the protein encoded by SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36.

Homologs of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 mean for example also bacterial, fungal and plant homologs, truncated sequences, single-stranded DNA or RNA of the coding and noncoding DNA sequence.

Homologs of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 also mean derivatives such as, for example, promoter variants. The promoters upstream of the nucleotide sequences detailed can be modified by one or more nucleotide exchanges, by insertion(s) and/or deletion(s) without the functionality or activity of the promoters being adversely affected, however. It is furthermore possible that the modification of the promoter sequence enhances their activity or that they are replaced entirely by more active promoters, including those from heterologous organisms.

The abovementioned nucleic acids and protein molecules with lysophosphatidic acid

acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase activity which are involved in the metabolism of lipids and fatty acids, PUFA cofactors and enzymes or in the transport of lipophilic compounds across membranes are used in the process according to the invention for the modulation of the production of PUFAs in transgenic organisms, advantageously in plants, such as maize, wheat, rye, oats, triticale, rice, barley, soybean, peanut, cotton, Linum species such as linseed or flax, Brassica species such as oilseed rape, canola and turnip rape, pepper, sunflower, borage, evening primrose and Tagetes, Solanaceae plants such as potato, tobacco, eggplant and tomato, Vicia species, pea, cassava, alfalfa, bushy plants (coffee, cacao, tea), Salix species, trees (oil palm, coconut) and perennial grasses and fodder crops, either directly (for example when the overexpression or optimization of a fatty acid biosynthesis protein has a direct effect on the yield, production and/or production efficiency of the fatty acid from modified organisms) and/or can have an indirect effect which nevertheless leads to an enhanced yield, production and/or production efficiency of the PUFAs or a reduction of undesired compounds (for example when the modulation of the metabolism of lipids and fatty acids, cofactors and enzymes leads to modifications of the yield, production and/or production efficiency or the composition of the desired compounds within the cells, which, in turn, can affect the production of one or more fatty acids).

The combination of various precursor molecules and biosynthesis enzymes leads to the production of various fatty acid molecules, which has a decisive effect on lipid composition, since polyunsaturated fatty acids (= PUFAs) are not only incorporated into triacylglycerol but also into membrane lipids.

Lipid synthesis can be divided into two sections: the synthesis of fatty acids and their binding to sn-glycerol-3-phosphate, and the addition or modification of a polar head group. Usual lipids which are used in membranes comprise phospholipids, glycolipids, sphingolipids and phosphoglycerides. Fatty acid synthesis starts with the conversion of acetyl-CoA into malonyl-CoA by acetyl-CoA carboxylase or into acetyl-ACP by acetyl transacylase. After a condensation reaction, these two product molecules together form acetoacetyl-ACP, which is converted via a series of condensation, reduction and dehydratization reactions so that a saturated fatty acid molecule with the desired chain length is obtained. The production of the unsaturated fatty acids from these molecules is catalyzed by specific desaturases, either aerobically by means of molecular oxygen or anaerobically (regarding the fatty acid synthesis in microorganisms, see F.C. Neidhardt et al. (1996) E. coli and Salmonella. ASM Press: Washington, D.C., pp. 612-636 and references cited therein; Lengeler et al. (Ed.) (1999) Biology of Procaryotes. Thieme: Stuttgart, New York, and the references therein, and Magnuson, K., et al. (1993) Microbiological Reviews 57:522-542 and the references therein). To undergo the further elongation steps, the resulting phospholipid-bound fatty acids must then be returned from the phospholipids to the fatty acid CoA ester pool. This is made possible by acyl-CoA:lysophospholipid acyltransferases. Moreover, these enzymes are capable of transferring the elongated fatty acids from the CoA esters back to the phospholipids. If appropriate, this reaction sequence can be followed repeatedly.

Examples of precursors for the biosynthesis of PUFAs are oleic acid, linoleic acid and linolenic acid. These C_{18}-carbon fatty acids must be elongated to C_{20} and C_{22} in order to obtain fatty acids of the eicosa and docosa chain type. With the aid of the lysophosphatidic acid acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases, lecithin cholesterol acyltransferases used in the process, advantageously in combination with acyl-CoA:lysophospholipid acyltransferases, desaturases such as Δ-4-, Δ-5-, Δ-6- and Δ-8-desaturases and/or Δ-5-, Δ-6-, Δ-9-elongases, arachidonic acid, eicosapentaenoic acid, docosapentaenoic acid or docosahexaenoic acid and various other long-chain PUFAs can be obtained, extracted and employed in various applications regarding foodstuffs, feedstuffs, cosmetics or pharmaceuticals. Preferably, C_{18}-, C_{20}-, C_{22}- and/or C_{24}-fatty acids with at least two, advantageously at least three, four, five or six, double bonds in the fatty acid molecule can be prepared using the abovementioned enzymes, to give preferably C_{20}-, C_{22}- and/or C_{24}-fatty acids with advantageously three, four or five double bonds in the fatty acid molecule. Desaturation may take place before or after elongation of the fatty acid in question. This is why the products of the desaturase activities and the further desaturation and elongation steps which are possible result in preferred PUFAs with a higher degree of desaturation, including a further elongation from C_{20}- to C_{22}-fatty acids, to fatty acids such as γ-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, stearidonic acid, eicosatetraenoic acid or eicosapentaenoic acid. Substrates of the lysophosphatidic acyltransferases, glycerol-3-phosphate acyltransferases, diacylglycerol acyltransferases or lecithin cholesterol acyltransferases in the process according to the invention are C_{18}-, C_{20}- or C_{22}-fatty acids such as, for example, linoleic acid, γ-linolenic acid, α-linolenic acid, dihomo-γ-linolenic acid, eicosatetraenoic acid or stearidonic acid. Preferred substrates are linoleic acid, γ-linolenic acid and/or α-linolenic acid, dihomo-γ-linolenic acid, arachidonic acid, eicosatetraenoic acid or eicosapentaenoic acid. The C_{18}-, C_{20}- or C_{22}-fatty acids with at least two double bonds in the fatty acid are obtained in the process according to the invention in the form of the free fatty acid or in the form of their esters, for example in the form of their glycerides.

The term "glyceride" is understood as meaning a glycerol esterified with one, two or three carboxyl radicals (mono-, di- or triglyceride). "Glyceride" is also understood as meaning a mixture of various glycerides. The glyceride or glyceride mixture may comprise further additions, for example free fatty acids, antioxidants, proteins, carbohydrates, vitamins and/or other substances.

For the purposes of the process of the invention, a "glyceride" is furthermore understood as meaning glycerol derivatives. In addition to the above-described fatty acid glycerides, these also include glycerophospholipids and glyceroglycolipids. Preferred examples which may be mentioned in this context are the glycerophospholipids such as lecithin (phosphatidylcholine), cardiolipin, phosphatidylglycerol, phosphatidylserine and alkylacylglycerophospholipids.

Furthermore, fatty acids must subsequently be translocated to various modification sites and incorporated into the triacylglycerol storage lipid. A further important step in

lipid synthesis is the transfer of fatty acids to the polar head groups, for example by glycerol fatty acid acyltransferase (see Frentzen, 1998, Lipid, 100(4-5):161-166).

For publications on plant fatty acid biosynthesis and on the desaturation, the lipid metabolism and the membrane transport of lipidic compounds, on beta-oxidation, fatty acid modification and cofactors, triacylglycerol storage and triacylglycerol assembly, including the references therein, see the following papers: Kinney, 1997, Genetic Engineering, Ed.: JK Setlow, 19:149-166; Ohlrogge and Browse, 1995, Plant Cell 7:957-970; Shanklin and Cahoon, 1998, Annu. Rev. Plant Physiol. Plant Mol. Biol. 49:611-641; Voelker, 1996, Genetic Engineering, Ed.: JK Setlow, 18:111-13; Gerhardt, 1992, Prog. Lipid R. 31:397-417; Gühnemann-Schäfer & Kindl, 1995, Biochim. Biophys Acta 1256:181-186; Kunau et al., 1995, Prog. Lipid Res. 34:267-342; Stymne et al., 1993, in: Biochemistry and Molecular Biology of Membrane and Storage Lipids of Plants, Ed.: Murata and Somerville, Rockville, American Society of Plant Physiologists, 150-158, Murphy & Ross 1998, Plant Journal. 13(1):1-16.

The PUFAs produced in the process comprise a group of molecules which higher animals are no longer capable of synthesizing and must therefore take up, or which higher animals are no longer capable of synthesizing themselves in sufficient quantity and must therefore take up additional quantities, although they are synthesized readily by other organisms such as bacteria; for example, cats are no longer capable of synthesizing arachidonic acid.

The term "lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase" comprises for the purposes of the invention proteins which participate in the biosynthesis of fatty acids and their homologs, derivatives and analogs. Phospholipids for the purposes of the invention are understood as meaning phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol and/or phosphatidylinositol, advantageously phosphatidylcholine. The terms lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase nucleic acid sequence(s) comprise nucleic acid sequences which code for a lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase and part of which may be a coding region and likewise corresponding 5' and 3' untranslated sequence regions. The terms production or productivity are known in the art and encompass the concentration of the fermentation product (compounds of the formula I) which is formed within a specific period of time and in a specific fermentation volume (for example kg of product per hour per liter). The term production efficiency comprises the time required for obtaining a specific production quantity (for example the time required by the cell to establish a certain throughput rate of a fine chemical). The term yield or product/carbon yield is known in the art and comprises the efficiency of the conversion of the carbon source into the product (i.e. the fine chemical). This is usually expressed for example

as kg of product per kg of carbon source. By increasing the yield or production of the compound, the amount of the molecules obtained of this compound, or of the suitable molecules of this compound obtained in a specific culture quantity over a specified period of time is increased. The terms biosynthesis or biosynthetic pathway are known in the art and comprise the synthesis of a compound, preferably of an organic compound, by a cell from intermediates, for example in a multi-step and strongly regulated process. The terms catabolism or catabolic pathway are known in the art and comprise the cleavage of a compound, preferably of an organic compound, by a cell to give catabolites (in more general terms, smaller or less complex molecules), for example in a multi-step and strongly regulated process. The term metabolism is known in the art and comprises the totality of the biochemical reactions which take place in an organism. The metabolism of a certain compound (for example the metabolism of a fatty acid) thus comprises the totality of the biosynthetic pathways, modification pathways and catabolic pathways of this compound in the cell which relate to this compound.

In a further embodiment, derivatives of the nucleic acid molecule according to the invention represented in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 code for proteins with at least 40%, advantageously from approximately 50 to 60%, preferably at least from approximately 60 to 70% and more preferably at least from approximately 70 to 80%, 80 to 90%, 90 to 95% and most preferably at least approximately 96%, 97%, 98%, 99% or more homology (= identity) with a complete amino acid sequence of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35 or SEQ ID NO: 37. The homology was calculated over the entire amino acid or nucleic acid sequence region. The program PileUp (J. Mol. Evolution., 25, 351-360, 1987, Higgins et al., CABIOS, 5 1989: 151–153) or the programs Gap and BestFit [Needleman and Wunsch (J. Mol. Biol. 48; 443-453 (1970) and Smith and Waterman (Adv. Appl. Math. 2; 482-489 (1981)], which are part of the GCG software packet [Genetics Computer Group, 575 Science Drive, Madison, Wisconsin, USA 53711 (1991)], were used for the sequence alignment. The sequence homology values which are indicated above as percentages were determined over the entire sequence region using the program BestFit and the following settings: Gap Weight: 8, Length Weight: 2.

Moreover, the invention comprises nucleic acid molecules which differ from one of the nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID

NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 (and parts thereof) owing to the degeneracy of the genetic code and which thus code for the same lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase as those encoded by the nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36.

In addition to the lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase nucleotide sequences shown in SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36, the skilled worker will recognize that DNA sequence polymorphisms which lead to changes in the amino acid sequences of the lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase may exist within a population. These genetic polymorphisms in the lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase gene may exist between individuals within a population owing to natural variation. These natural variants usually bring about a variance of 1 to 5% in the nucleotide sequence of the lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase gene. Each and every one of these nucleotide variations and resulting amino acid polymorphisms in the lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase which are the result of natural variation and do not modify the functional activity of are to be encompassed by the invention.

Owing to their homology to the lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase nucleic acids disclosed here, nucleic acid molecules which are advantageous for the process according to the invention can be isolated following standard hybridization techniques under stringent hybridization conditions, using the sequences or part thereof as hybridization probe. In this context it is possible, for example, to use isolated nucleic acid molecules which are at least 15 nucleotides in length and which hybridize under stringent conditions with the nucleic acid molecules which comprise a nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13,

SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36. Nucleic acids with at least 25, 50, 100, 250 or more nucleotides can also be used. The term "hybridizes under stringent conditions" as used in the present context is intended to describe hybridization and washing conditions under which nucleotide sequences with at least 60% homology to one another usually remain hybridized with one another. Conditions are preferably such that sequences with at least approximately 65%, preferably at least approximately 70% and especially preferably at least approximately 75% or more homology to one another usually remain hybridized with one another. These stringent conditions are known to the skilled worker and can be found in Current Protocols in Molecular Biology, John Wiley & Sons, N. Y. (1989), 6.3.1-6.3.6. A preferred nonlimiting example of stringent hybridization conditions is hybridizations in 6 x sodium chloride/sodium citrate (= SSC) at approximately 45°C, followed by one or more washing steps in 0.2 x SSC, 0.1% SDS at 50 to 65°C. The skilled worker knows that these hybridization conditions differ depending on the type of nucleic acid and, for example when organic solvents are present, regarding temperature and buffer concentration. Under "standard hybridization conditions", for example, the temperature is, depending on the type of nucleic acid, between 42°C and 58°C in aqueous buffer with a concentration of 0.1 to 5 x SSC (pH 7.2). If organic solvent, for example 50% formamide, is present in the abovementioned buffer, the temperature under standard conditions is approximately 42°C. The hybridization conditions for DNA:DNA hybrids, for example, are preferably 0.1 x SSC and 20°C to 45°C, preferably 30°C to 45°C. The hybridization conditions for DNA:RNA hybrids are, for example, preferably 0.1 x SSC and 30°C to 55°C, preferably 45°C to 55°C. The abovementioned hybridization temperatures are determined by way of example for a nucleic acid with approximately 100 bp (= base pairs) in length and with a G + C content of 50% in the absence of formamide. The skilled worker knows how to determine the required hybridization conditions on the basis of the abovementioned textbooks or textbooks such as Sambrook et al., "Molecular Cloning", Cold Spring Harbor Laboratory, 1989; Hames and Higgins (Ed.) 1985, "Nucleic Acids Hybridization: A Practical Approach", IRL Press at Oxford University Press, Oxford; Brown (Ed.) 1991, "Essential Molecular Biology: A Practical Approach", IRL Press at Oxford University Press, Oxford.

In order to determine the percentage of homology (= identity) of two amino acid sequences (for example one of the sequences of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35 or SEQ ID NO: 37) or of two nucleic acids (for example SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID

NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36), the sequences are written one under the other for an optimal comparison (for example, gaps may be introduced into the sequence of a protein or of a nucleic acid in order to generate an optimal alignment with the other protein or the other nucleic acid). Then, the amino acid residues or nucleotides at the corresponding amino acid positions or nucleotide positions are compared. If a position in a sequence is occupied by the same amino acid residue or the same nucleotide as the corresponding position in the other sequence, then the molecules are homologous at this position (i.e. amino acid or nucleic acid "homology" as used in the present context corresponds to amino acid or nucleic acid "identity"). The percentage of homology between the two sequences is a function of the number of identical positions which the sequences share (i.e. % homology = number of identical positions/total number of positions x 100). The terms homology and identity are therefore to be considered as synonymous. The programs and algorithms used are described above.

An isolated nucleic acid molecule which codes for a lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase which is homologous to a protein sequence of SEQ ID NO: 2, SEQ ID NO: 5, SEQ ID NO: 8, SEQ ID NO: 10, SEQ ID NO: 12, SEQ ID NO: 15, SEQ ID NO: 17, SEQ ID NO: 19, SEQ ID NO: 21, SEQ ID NO: 23, SEQ ID NO: 25, SEQ ID NO: 27, SEQ ID NO: 29, SEQ ID NO: 31, SEQ ID NO: 33, SEQ ID NO: 35 or SEQ ID NO: 37 can be generated by introducing one or more nucleotide substitutions, additions or deletions into a nucleotide sequence of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 so that one or more amino acid substitutions, additions or deletions are introduced into the protein which is encoded. Mutations in one of the sequences of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36 can be introduced by standard techniques such as site-specific mutagenesis and PCR-mediated mutagenesis. It is preferred to generate conservative amino acid substitutions in one or more of the predicted nonessential amino acid residues. In a "conservative amino acid substitution", the amino acid residue is replaced by an amino acid residue with a similar side chain. Families of amino acid residues with similar side chains have been defined in the art. These families comprise amino acids with basic side chains (for example lysine, arginine, histidine), acidic side chains (for example aspartic acid, glutamic acid), uncharged polar side chains (for example glycine, asparagine, glutamine, serine, threonine, tyrosine,

cysteine), unpolar side chains (for example alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (for example threonine, valine, isoleucine) and aromatic side chains (for example tyrosine, phenylalanine, tryptophan, histidine). A predicted nonessential amino acid residue in a lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase is thus preferably replaced by another amino acid residue from the same family of side chains. In another embodiment, the mutations can, alternatively, be introduced randomly over all or part of the sequence coding for lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase, for example by saturation mutagenesis, and the resulting mutants can be screened by the herein-described lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase activity in order to identify mutants which have retained the lysophosphatidic acid acyltransferase, glycerol-3-phosphate acyltransferase, diacylglycerol acyltransferase or lecithin cholesterol acyltransferase activity. Following the mutagenesis of one of the sequences of SEQ ID NO: 1, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 9, SEQ ID NO: 11, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 16, SEQ ID NO: 18, SEQ ID NO: 20, SEQ ID NO: 22, SEQ ID NO: 24, SEQ ID NO: 26, SEQ ID NO: 28, SEQ ID NO: 30, SEQ ID NO: 32, SEQ ID NO: 34 or SEQ ID NO: 36, the protein which is encoded can be expressed recombinantly, and the activity of the protein can be determined, for example using the tests described in the present text.

The present invention is illustrated in greater detail by the examples which follow, which are not to be construed as limiting. The content of all of the references, patent applications, patents and published patent applications cited in the present patent application is herewith incorporated by reference.

Examples

Example 1: General methods

a) General cloning methods:

Cloning methods such as, for example, restriction cleavages, agarose gel electrophoresis, purification of DNA fragments, transfer of nucleic acids to nitrocellulose and nylon membranes, linking of DNA fragments, transformation of Escherichia coli and yeast cells, cultivation of bacteria and sequence analysis of recombinant DNA were carried out as described in Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6) or Kaiser, Michaelis and Mitchell (1994) "Methods in Yeast Genetics" (Cold Spring Harbor Laboratory Press: ISBN 0-87969-451-3).

b) Chemicals

Unless stated otherwise in the text, the chemicals used were obtained in analytical-grade quality from Fluka (Neu-Ulm, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), Serva (Heidelberg, Germany) and Sigma (Deisenhofen, Germany). Solutions were prepared using purified, pyrogen-free water, referred to as H_2O hereinbelow, from a Milli-Q Water System water purification system (Millipore, Eschborn, Germany). Restriction endonucleases, DNA-modifying enzymes and molecular-biological kits were obtained from AGS (Heidelberg, Germany), Amersham (Brunswick, Germany), Biometra (Göttingen, Germany), Boehringer (Mannheim, Germany), Genomed (Bad Oeynhausen, Germany), New England Biolabs (Schwalbach/Taunus, Germany), Novagen (Madison, Wisconsin, USA), Perkin-Elmer (Weiterstadt, Germany), Pharmacia (Freiburg, Germany), Qiagen (Hilden, Germany) and Stratagene (Amsterdam, the Netherlands). Unless stated otherwise, they were used according to the manufacturer's instructions.

c) Cloning and expression of desaturases and elongases

The Escherichia coli strain XL1 Blue MRF' kan (Stratagene) was used for subcloning Δ-6-desaturase from Physcomitrella patens. This gene was functionally expressed using the Saccharomyces cerevisiae strain INVSc 1 (Invitrogen Co.). E. coli was cultured in Luria-Bertani broth (LB, Duchefa, Haarlem, the Netherlands) at 37°C. If necessary, ampicillin (100 mg/liter) was added and 1.5% (w/v) agar was added for solid LB media. S. cerevisiae was cultured at 30°C either in YPG medium or in complete minimal medium without uracil (CMdum; see in: Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., Struhl, K., Albright, L.B., Coen, D.M., and Varki, A. (1995) Current Protocols in Molecular Biology, John Wiley & Sons, New York) with either 2% (w/v) raffinose or glucose. For solid media, 2% (w/v) Bacto™-Agar (Difco) were added. The plasmids used for cloning and expression are pUC18 (Pharmacia) and pYES2 (Invitrogen Co.).

d) Cloning and expression of PUFA-specific desaturases and elongases

For expression in plants, cDNA clones of SEQ ID NO: 46 (Physcomitrella patens Δ-6-desaturase), 48 (Physcomitrella patens Δ-6-elongase) or 50 (Phaeodactylum tricornutum Δ-5-desaturase) were modified so as for only the coding region to be amplified by means of polymerase chain reaction with the aid of two oligonucleotides. Care was taken here to observe a consensus sequence upstream of the start codon, for efficient translation. To this end, either the ATA or the AAA base sequence was chosen and inserted into the sequence upstream of the ATG [Kozak, M. (1986) Point mutations define a sequence flanking the AUG initiator codon that modulates translation by eukaryotic ribosomes, Cell 44, 283-2929]. In addition, a restriction cleavage site was introduced upstream of this consensus triplet, which must be compatible with the cleavage site of the target vector into which the fragment is to be cloned and with the aid of which gene expression is to be carried out in micro-organisms or plants.

The PCR reaction was carried out in a thermocycler (Biometra), using plasmid DNA as

template and Pfu DNA polymerase (Stratagene) and the following temperature program: 3 min at 96°C, followed by 30 cycles of 30 s at 96°C, 30 s at 55°C and 2 min at 72°C, 1 cycle of 10 min at 72°C and stop at 4°C. The annealing temperature was varied depending on the oligonucleotides chosen. A synthesis time of about one minute per kilobase pair of DNA has to be taken as starting point. Other parameters which influence the PCR, such as, for example, Mg ions, salt, DNA polymerase etc., are familiar to the skilled worker in the field and may be varied as required.

The correct size of the amplified DNA fragment was confirmed by means of agarose-TBE gel electrophoresis. The amplified DNA was extracted from the gel using the QIAquick gel extraction kit (QIAGEN) and ligated into the SmaI restriction site of the dephosphorylated pUC18 vector, using the Sure Clone Ligations Kit (Pharmacia), resulting in the pUC derivatives. After transformation of E. coli XL1 Blue MRF' kan a DNA minipreparation [Riggs, M.G., & McLachlan, A. (1986) A simplified screening procedure for large numbers of plasmid mini-preparation. BioTechniques 4, 310-313] of ampicillin-resistant transformants was carried out, and positive clones were identified by means of BamHI restriction analysis. The sequence of the cloned PCR product was confirmed by means of resequencing using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer, Weiterstadt, Germany).

e) Transformation of Agrobacterium

Unless described otherwise, Agrobacterium-mediated plant transformation was carried out with the aid of an Agrobacterium tumefaciens strain, as by Deblaere et al. (1984, Nucl. Acids Res. 13, 4777-4788).

f) Plant transformation

Unless described otherwise, Agrobacterium-mediated plant transformation was carried out using standard transformation and regeneration techniques (Gelvin, Stanton B., Schilperoort, Robert A., Plant Molecular Biology Manual, 2nd ed., Dordrecht: Kluwer Academic Publ., 1995, in Sect., Ringbuc Zentrale Signatur: BT11-P ISBN 0-7923-2731-4; Glick, Bernard R., Thompson, John E., Methods in Plant Molecular Biology and Biotechnology, Boca Raton: CRC Press, 1993, 360 S., ISBN 0-8493-5164-2).

According thereto, it is possible to transform, for example, oilseed rape by means of cotyledon or hypocotyl transformation (Moloney et al., Plant Cell 8 (1989) 238-242; De Block et al., Plant Physiol. 91 (1989) 694-701). The use of antibiotics for the selection of agrobacteria and plants depends on the binary vector used for transformation and the Agrobacterium strain. Normally, oilseed rape is selected using kanamycin as selectable plant marker.

The transformation of soybean may be carried out using, for example, a technique described in EP-A-0 0424 047 (Pioneer Hi-Bred International) or in EP-A-0 0397 687, US 5,376,543, US 5,169,770 (University Toledo).

The transformation of plants using particle bombardment, polyethylene glycol-mediated DNA uptake or via the silicon carbonate fiber technique is described, for example, by Freeling and Walbot "The maize handbook" (1993) ISBN 3-540-97826-7, Springer Verlag New York).

Unless described otherwise, Agrobacterium-mediated gene transfer into linseed (Linum usitatissimum) was carried out by the technique as described in Mlynarova et al. [(1994) Plant Cell Report 13:282-285].

g) Plasmids for plant transformation

Binary vectors based on the vectors pBinAR (Höfgen and Willmitzer, Plant Science 66 (1990) 221-230) or pGPTV (Becker et al. 1992, Plant Mol. Biol. 20:1195-1197) were used for plant transformation. The binary vectors which comprise the nucleic acids to be expressed are constructed by ligating the cDNA in sense orientation into the T-DNA. 5' of the cDNA, a plant promoter activates cDNA transcription. A polyadenylation sequence is located 3' of the cDNA. The binary vectors may carry different marker genes such as, for example, the acetolactate synthase gene (AHAS or ALS) [Ott et al., J. Mol. Biol. 1996, 263:359-360] which imparts a resistance to the imidazolinones or the nptII marker gene which codes for a kanamycin resistance imparted by neomycin phosphotransferase.

Tissue-specific expression of the nucleic acids can be achieved using a tissue-specific promoter. Unless described otherwise, the LeB4 or the USP promoter or the phaseolin promoter was cloned 5' of the cDNA. Terminators used were the NOS terminator and the OCS terminator (see figure 1). Figure 1 depicts a vector map of the vector used for expression, pSUN3CeLPLAT.

It is also possible to use any other seed-specific promoter element such as, for example, the napin or arcelin promoter (Goossens et al. 1999, Plant Phys. 120(4):1095-1103 and Gerhardt et al. 2000, Biochimica et Biophysica Acta 1490(1-2):87-98).

The CaMV-35S promoter or a v-ATPase C1 promoter can be used for constitutive expression in the whole plant.

The nucleic acids used in the process which code for acyl-CoA:lysophospholipid acyltransferases; desaturases or elongases were cloned into a binary vector one after the other by constructing a plurality of expression cassettes, in order to mimic the metabolic pathway in plants.

Within an expression cassette, the protein to be expressed may be targeted into a cellular compartment by using a signal peptide, for example for plastids, mitochondria or the endoplasmic reticulum (Kermode, Crit. Rev. Plant Sci. 15, 4 (1996) 285-423). The signal peptide is cloned 5' of and in-frame with the cDNA in order to achieve the subcellular localization of the fusion protein.

Examples of multiexpression cassettes were disclosed in DE 102 19 203 and are given again below.

i.) Promoter-terminator cassettes

Expression cassettes consist of at least two functional units such as a promoter and a terminator. Further desired gene sequences such as targeting sequences, coding regions of genes or parts thereof etc. may be inserted between promoter and terminator. To construct the expression cassettes, promoters and terminators (USP promoter: Baeumlein et al., Mol Gen Genet, 1991, 225 (3):459-67); OCS terminator: Gielen et al. EMBO J. 3 (1984) 835ff.) were isolated with the aid of the polymerase chain reaction and tailor-made with flanking sequences of choice on the basis of synthetic oligonucleotides.

Examples of oligonucleotides which may be used are the following:

USP1 upstream:
- CCGGAATTCGGCGCGCCGAGCTCCTCGAGCAAATTTACACATTGCCA -

USP2 upstream:
- CCGGAATTCGGCGCGCCGAGCTCCTCGAGCAAATTTACACATTGCCA -

USP3 upstream:
- CCGGAATTCGGCGCGCCGAGCTCCTCGAGCAAATTTACACATTGCCA -

USP1 downstream:
- AAAACTGCAGGCGGCCGCCCACCGCGGTGGGCTGGCTATGAAGAAATT -

USP2 downstream:
- CGCGGATCCGCTGGCTATGAAGAAATT -

USP3 downstream:
- TCCCCCGGGATCGATGCCGGCAGATCTGCTGGCTATGAAGAAATT -

OCS1 upstream:
- AAAACTGCAGTCTAGAAGGCCTCCTGCTTTAATGAGATAT -

OCS2 upstream:
- CGCGGATCCGATATCGGGCCCGCTAGCGTTAACCCTGCTTTAATGAGATAT -

OCS3 upstream:
- TCCCCCGGGCCATGGCCTGCTTTAATGAGATAT -

OCS1 downstream:
- CCCAAGCTTGGCGCGCCGAGCTCGAATTCGTCGACGGACAATCAGTAAATTGA -

OCS2 downstream:
- CCCAAGCTTGGCGCGCCGAGCTCGAATTCGTCGACGGACAATCAGTAAATTGA -

OCS3 downstream:
- CCCAAGCTTGGCGCGCCGAGCTCGTCGACGGACAATCAGTAAATTGA -

The methods are known to the skilled worker in the field and are well known from the literature.

In a first step, a promoter and a terminator were amplified via PCR. The terminator was then cloned into a recipient plasmid and, in a second step, the promoter was inserted upstream of the terminator. As a result, an expression cassette was cloned into the basic plasmid. The plasmids pUT1, 2 and 3 were thus generated on the basis of the pUC19 plasmid.

The corresponding constructs or plasmids are defined in SEQ ID NO: 52, 53 and 54. They comprise the USP promoter and the OCS terminator. Based on these plasmids, the construct pUT12 was generated by cutting pUT1 by means of SalI/ScaI and pUT2 by means of XhoI/ScaI. The fragments comprising the expression cassettes were ligated and transformed into E. coli XL1 blue MRF. After isolating ampicillin-resistant colonies, DNA was prepared and those clones which comprise two expression cassettes were identified by restriction analysis. The XhoI/SalI ligation of compatible ends has eliminated here the two cleavage sites, XhoI and SalI, between the expression cassettes. The resulting plasmid, pUT12, is indicated in SEQ ID NO: 55. Subsequently, pUT12 was cut again by means of SalI/ScaI and pUT3 was cut by means of XhoI/ScaI. The fragments comprising the expression cassettes were ligated and transformed into E. coli XLI blue MRF. After isolation from ampicillin-resistant colonies, DNA was again prepared, and those clones which comprise three expression cassettes were identified by restriction analysis. In this manner, a set of multiexpression cassettes was produced which can be utilized for insertion of desired DNA and which is described in table 1 and which moreover can incorporate further expression cassettes.

Said cassettes comprise the following elements:

Table 1

PUC19 derivative	Cleavage sites upstream of the USP promoter	Multiple cloning cleavage sites	Cleavage sites downstream of the OCS terminator
PUT1	EcoRI/AscI/ SacI/XhoI	BstXI/NotI/ PstI/XbaI/StuI	SalI/EcoRI/ SacI/AscI/ HindIII
PUT2	EcoRI/AscI/ SacI/XhoI	BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/ SacI/AscI/ HindIII
PUT3	EcoRI/AscI/ SacI/XhoI	BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/ AscI/HindIII
PUT12 double expression cassette	EcoRI/AscI/ SacI/XhoI	BstXI/NotI/ PstI/XbaI/StuI and BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/ SacI/AscI/ HindIII
PUT123 triple expression cassette	EcoRI/AscI/ SacI/XhoI	1. BstXI/NotI/ PstI/XbaI/StuI and 2. BamHI/EcoRV/ ApaI/NheI/ HpaI and 3. BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/AscI/HindIII

Furthermore, further multiexpression cassettes may be generated, as described and as specified in more detail in table 2, with the aid of the

i) USP promoter or with the aid of the

ii) 700 base pair 3' fragment of the LeB4 promoter or with the aid of the

iii) DC3 promoter and employed for seed-specific gene expression.

The DC3 promoter is described in Thomas, Plant Cell 1996, 263:359-368 and consists merely of the region from -117 to +26, which is why it therefore constitutes one of the smallest known seed-specific promoters. The expression cassettes may comprise several copies of the same promoter or else be constructed via three different promoters.

Advantageously used polylinker- or polylinker-terminator-polylinkers can be found in the sequences SEQ ID NO: 60 to 62.

Table 2: Multiple expression cassettes

Plasmid name of the pUC19 derivative	Cleavage sites upstream of the particular promoter	Multiple cloning cleavage sites	Cleavage sites downstream of the OCS terminator
pUT1 (pUC19 with USP-OCS1)	EcoRI/AscI/SacI/XhoI	(1) BstXI/NotI/PstI/ XbaI/StuI	SalI/EcoRI/SacI/AscI/ HindIII
PDCT (pUC19 with DC3-OCS)	EcoRI/AscI/SacI/XhoI	(2) BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/SacI/AscI/ HindIII
PleBT (pUC19 with LeB4(700)-OCS)	EcoRI/AscI/SacI/XhoI	(3) BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/AscI/HindIII
PUD12 (pUC 19 with USP-OCS1 and with DC3-OCS)	EcoRI/AscI/SacI/XhoI	(1) BstXI/NotI/ PstI/XbaI/StuI and (2) BamHI/EcoRV/ ApaI/NheI/ HpaI	SalI/EcoRI/SacI/AscI/ HindIII
PUDL123 Triple expression cassette (pUC19 with USP/DC3 and LeB4-700)	EcoRI/AscI/SacI/XhoI	(1) BstXI/NotI/ PstI/XbaI/StuI and (2) BamHI/ (EcoRV*)/ApaI/NheI/ HpaI and (3) BglII/NaeI/ ClaI/SmaI/NcoI	SalI/SacI/AscI/HindIII

* EcoRV cleavage site cuts in the 700 base pair fragment of the LeB4 promoter (LeB4-700)

Further promoters for multigene constructs can be generated analogously, in particular by using the

a) 2.7 kB fragment of the LeB4 promoter or with the aid of the

b) phaseolin promoter or with the aid of the

c) constitutive v-ATPase c1 promoter.

It may be particularly desirable to use further particularly suitable promoters for constructing seed-specific multiexpression cassettes, such as, for example, the napin promoter or the arcelin-5 promoter.

Further vectors which can be utilized in plants and which have one or two or three promoter-terminator expression cassettes can be found in the sequences SEQ ID NO: 63 to SEQ ID NO: 68.

ii.) Generation of expression constructs which comprise promoter, terminator and desired gene sequence for the expression of PUFA genes in plant expression cassettes.

The Δ-6-elongase Pp_PSE1 is first inserted into the first cassette in pUT123 via BstXI and XbaI. Then, the moss Δ-6-desaturase (Pp_des6) is inserted via BamHI/NaeI into the second cassette and, finally, the Phaeodactylum Δ-5-desaturase (Pt_des5) is inserted via BglII/NcoI into the third cassette (see SEQ ID NO: 56). The triple construct is named pARA1. Taking into consideration sequence-specific restriction cleavage sites, further expression cassettes, as set out in table 3 and referred to as pARA2, pARA3 and pARA4, may be generated.

Table 3: Combinations of desaturases and elongases

Gene plasmid	Δ-6-Desaturase	Δ-5-Desaturase	Δ-6-Elongase
pARA1	Pp_des6	Pt_des5	Pp_PSE1
pARA2	Pt_des6	Pt_des5	Pp_PSE1
pARA3	Pt_des6	Ce_des5	Pp_PSE1
PARA4	Ce_des6	Ce_des5	Ce_PSE1

des5 = PUFA-specific Δ-5-desaturase
des6 = PUFA-specific Δ-6-desaturase
PSE = PUFA-specific Δ-6-elongase
Pt_des5 = Δ-5-desaturase from Phaeodactylum tricornutum
Pp_des6 or Pt_des6 = Δ-6-desaturase from Physcomitrella patens or Phaeodactylum tricornutum
Pp = Physcomitrella patens, Pt = Phaeodactylum tricornutum
Pp_PSE1 = Δ-6-elongase from Physcomitrella patens
Pt_PSE1 = Δ-6-elongase from Phaeodactylum tricornutum
Ce_des5 = Δ-5-desaturase from Caenorhabditis elegans (Genbank Acc. No. AF078796)
Ce_des6 = Δ-6-desaturase from Caenorhabditis elegans (Genbank Acc. No. AF031477, bases 11-1342)
Ce_PSE1 = Δ-6-elongase from Caenorhabditis elegans (Genbank Acc. No. AF244356, bases 1-867)

Further desaturases or elongase gene sequences may also be inserted into expression cassettes of the type described, such as, for example, Genbank Acc. No. AF231981, NM_013402, AF206662, AF268031, AF226273, AF110510 or AF110509.

iii.) Transfer of expression cassettes into vectors for the transformation of Agrobacterium tumefaciens and for the transformation of plants

The constructs thus generated were inserted into the binary vector pGPTV by means of AscI. For this purpose, the multiple cloning sequence was extended by an AscI cleavage site. For this purpose, the polylinker was synthesized de novo in the form of two double-stranded oligonucleotides, with an additional AscI DNA sequence being inserted. The oligonucleotide was inserted into the pGPTV vector by means of EcoRI

and HindIII. The cloning techniques required are known to the skilled worker and may readily be found in the literature as described in example 1.

The nucleic acid sequences for Δ-5-desaturase (SEQ ID NO: 50), Δ-6-desaturase (SEQ ID NO: 46) and Δ-6-elongase (SEQ ID NO: 48), which were used for the experiments described below, were the sequences from Physcomitrella patens and Phaeodactylum tricornutum. The corresponding amino acid sequences can be found in the sequences SEQ ID NO: 47, SEQ ID NO: 49 and SEQ ID NO: 51. A vector which comprises all of the abovementioned genes is indicated in SEQ ID NO: 56. The corresponding amino acid sequences of the genes can be found in SEQ ID NO: 57, SEQ ID NO: 58 and SEQ ID NO: 59.

Example 2: Cloning and characterization of the ceLPLATs (SEQ ID NO: 38-44)

a) Database search

The ceLPLATs (= acyl-CoA:lysophospholipid acyltransferase from Caenorhabditis elegans) were identified by sequence comparisons with known LPA-ATs. The search was restricted to the nematode genome (*Caenorhabditis elegans*) with the aid of the BLAST-Psi algorithm (Altschul et al., J. Mol. Biol. 1990, 215: 403-410), since this organism synthesizes LCPUFAs. The probe employed in the sequence comparison was an LPAAT protein sequence from *Mus musculus* (MsLPAAT Accession No. NP_061350). LPLAT catalyzes, by a reversible transferase reaction, the ATP-independent synthesis of acyl-CoAs from phospholipids with the aid of CoA as cofactor (Yamashita et al., J. Biol. Chem. 2001, 20: 26745-26752). Sequence comparisons enabled two putative ceLPLAT sequences to be identified (Accession No. *T06E8.1* and *F59F4.4*). The identified sequences are most similar to each other and to MsLPAATs (figure 2). The alignment was generated using the Clustal program.

b) Cloning of the CeLPLATs

Primer pairs were synthesized on the basis of the ceLPLAT nucleic acid sequences (table 4) and the corresponding cDNAs were isolated from a *C. elegans* cDNA library by means of PCR processes. The respective primer pairs were selected so as to carry, apart from the start codon, the yeast consensus sequence for high-efficiency translation (Kozak, Cell 1986, 44:283-292). The LPLAT cDNAs were amplified in each case using 2 µl of cDNA-library solution as template, 200 µM dNTPs, 2.5 U of "proof-reading" *pfu* polymerase and 50 pmol of each primer in a total volume of 50 µl. The conditions for the PCR were as follows: first denaturation at 95°C for 5 minutes, followed by 30 cycles at 94°C for 30 seconds, 58°C for one minute and 72°C for 2 minutes, and a final extension step at 72°C for 10 minutes. The sequence of the LPLAT cDNAs was confirmed by DNA sequencing.

Table 4: Nucleotide sequences of the PCR primers for cloning CeLPLATs

Primer	Nucleotide sequence
5' T06E8.1f*	5' ACATAATGGAGAACTTCTGGTCGATCGTC 3'
3' T06E8.1r*	5' TTACTCAGATTTCTTCCCGTCTTT 3'
5' F59F4.4f*	5' ACATAATGACCTTCCTAGCCATATTA 3'
3' F59F4.4r*	5' TCAGATATTCAAATTGGCGGCTTC 3'

* f: forward, r: reverse

Example 3: Analysis of the effect of the recombinant proteins on production of the desired product

a) Possible preparation methods

The effect of genetic modification in fungi, algae, ciliates or, as described in the examples hereinabove, on the production of the polyunsaturated fatty acids in yeasts, or in plants may be determined by growing the modified microorganisms or the modified plant under suitable conditions (such as those described above) and studying the medium and/or the cellular components for increased production of the lipids or fatty acids. These analytical techniques are known to the skilled worker and comprise spectroscopy, thin-layer chromatography, various types of staining methods, enzymic and microbiological methods and analytical chromatography such as high-performance liquid chromatography (see, for example, Ullmann, Encyclopedia of Industrial Chemistry, vol. A2, pp. 89-90 and pp. 443-613, VCH: Weinheim (1985); Fallon, A., et al., (1987) "Applications of HPLC in Biochemistry" in: Laboratory Techniques in Biochemistry and Molecular Biology, vol. 17; Rehm et al. (1993) Biotechnology, vol. 3, chapter III: "Product recovery and purification", pp. 469-714, VCH: Weinheim; Belter, P.A., et al. (1988) Bioseparations: downstream processing for Biotechnology, John Wiley and Sons; Kennedy, J.F., and Cabral, J.M.S. (1992) Recovery processes for biological Materials, John Wiley and Sons; Shaeiwitz, J.A., and Henry, J.D. (1988) Biochemical Separations, in: Ullmann's Encyclopedia of Industrial Chemistry, vol. B3; chapter 11, pp. 1-27, VCH: Weinheim; and Dechow, F.J. (1989) Separation and purification techniques in biotechnology, Noyes Publications).

Apart from the abovementioned methods for detecting fatty acids in yeasts, plant lipids are extracted from plant material as described by Cahoon et al. (1999) Proc. Natl. Acad. Sci. USA 96 (22):12935-12940, and Browse et al. (1986) Analytic Biochemistry 152:141-145. The qualitative and quantitative analysis of lipids or fatty acids is

described in Christie, William W., Advances in Lipid Methodology, Ayr/Scotland: Oily Press (Oily Press Lipid Library; 2); Christie, William W., Gas Chromatography and Lipids. A Practical Guide - Ayr, Scotland: Oily Press, 1989, Repr. 1992, IX, 307 S. (Oily Press Lipid Library; 1); "Progress in Lipid Research, Oxford: Pergamon Press, 1 (1952) - 16 (1977) under the title: Progress in the Chemistry of Fats and Other Lipids CODEN.

Thus, fatty acids or triacylglycerol (= TAG, abbreviations indicated in brackets) may be analyzed, for example, by means of fatty acid methyl esters (= FAME), gas liquid chromatography-mass spectrometry (= GC-MS) or thin-layer chromatography (TLC).

Unequivocal proof of the presence of fatty acid products may be obtained by means of analyzing recombinant organisms following standard analytical procedures: GC, GC-MS or TLC, as variously described by Christie and references therein (1997, in: Advances on Lipid Methodology, fourth ed.: Christie, Oily Press, Dundee, 119-169; 1998, Gaschromatographie-Massenspektrometrie-Verfahren [Gas chromatography-mass spectrometry methods], Lipide 33:343-353).

The plant material to be analyzed may for this purpose be disrupted either by sonification, glass milling, liquid nitrogen and grinding or via other applicable processes. After the material has been disrupted, it is then centrifuged. The sediment is then resuspended in distilled water, heated at 100°C for 10 min, cooled on ice and centrifuged again, followed by extraction in 0.5 M sulfuric acid in methanol containing 2% dimethoxypropane for 1 h at 90°C, leading to hydrolyzed oil and lipid compounds which result in transmethylated lipids. These fatty acid methyl esters may then be extracted in petroleum ether and finally be subjected to GC analysis using a capillary column (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 µm, 0.32 mm), with a temperature gradient of between 170°C and 240°C for 20 min and at 240°C for 5 min. The identity of the resulting fatty acid methyl esters can be defined using standards available from commercial sources (i.e. Sigma).

In the case of fatty acids for which no standards are available, the identity may be shown via derivatization and subsequent GC-MS analysis. For example, the localization of triple-bond fatty acids is shown via GC-MS after derivatization with 4,4-dimethoxyoxazoline derivatives (Christie, 1998, see above).

b) Fatty acid analysis in plants

Total fatty acids were extracted from plant seeds and analyzed by means of gas chromatography.

The seeds were taken up with 1% sodium methoxide in methanol and incubated at RT (approx. 22°C) for 20 min. This was followed by washing with NaCl solution and taking up the FAMEs in 0.3 ml of heptane.

The samples were fractionated on a ZEBRON-ZB Wax capillary column (30 m, 0.32 mm, 0.25 µm; Phenomenex) in a Hewlett Packard 6850 gas chromatograph with

flame ionization detector. The oven temperature was programmed from 70°C (hold for 1 min) to 200°C at a rate of 20°C/min, then to 250°C (hold for 5 min) at a rate of 5°C/min and finally to 260°C at a rate of 5°C/min. The carrier gas used was nitrogen (4.5 ml/min at 70°C). The fatty acids were identified by comparison with retention times of FAME standards (SIGMA).

Example 4: Functional characterization of CeLPLATs in yeast

a) Heterologous expression in Saccharomyces cerevisiae

To characterize the function of the *C. elegans* CeLPLATs (SEQ ID NO: 38-44), the open reading frames of the particular cDNAs were cloned downstream of the galactose-inducible GAL1 promoter of pYes2.1Topo, using the pYes2.1TOPO TA Expression Kit (Invitrogen), resulting in pYes2-T06E8.1 and pYes2-F59F4.4.

Since expression of the CeLPLATs should result in an efficient exchange of the acyl substrates, the double construct pESCLeu-PpD6-Pse1 which includes the open reading frames of a Δ6-desaturase (PpD6) and a Δ6-elongase (PSE1) from *Physcomitrella patens* (see DE 102 19 203) was also prepared. The nucleic acid sequence of said Δ6-desaturase (PpD6) and said Δ6-elongase (Pse1) are indicated in each case in SEQ ID NO: 46 and SEQ ID NO: 48. The corresponding amino acid sequences can be found in SEQ ID NO: 47 and SEQ ID NO: 49.

The *Saccharomyces cerevisiae* strains C13ABYS86 (protease-deficient) and INVSc1 were transformed simultaneously with the vectors pYes2-T06E8.1 and pESCLeu-PpD6-Pse1 and, respectively, pYes2-F59F4.4 and pESCLeu-PpD6-Pse1 by means of a modified PEG/lithium acetate protocol. The control used was a yeast which was transformed with the pESCLeu-PpD6-Pse1 vector and the empty vector pYes2. The transformed yeasts were selected on complete minimal medium (CMdum) agar plates containing 2% glucose but no uracil or leucine. After selection, 4 transformants, two pYes2-T06E8.1/pESCLeu-PpD6-Pse1 and two pYes2-F59F4.4/pESCLeu-PpD6-Pse1 and one pESCLeu-PpD6-Pse1/pYes2 were selected for further functional expression. The experiments described were also carried out in the yeast strain INVSc1.

In order to express the CeLPAATs, precultures of in each case 2 ml of CMdum liquid medium containing 2% (w/v) raffinose but no uracil or leucine were first inoculated with the selected transformants and incubated at 30°C, 200 rpm, for 2 days. 5 ml of CMdum liquid medium (without uracil and leucine) containing 2% raffinose, 1% (v/v) Tergitol NP-40 and 250 µM linoleic acid ($18:2^{\Delta 9,12}$) or linolenic acid ($18:3^{\Delta 9,12,15}$) were then inoculated with the precultures to an OD_{600} of 0.08. Expression was induced at an OD_{600} of 0.2-0.4 by adding 2% (w/v) galactose. The cultures were incubated at 20°C for a further 48 h.

Fatty acid analysis

The yeast cells from the main cultures were harvested by centrifugation (100 x g, 10 min, 20°C) and washed with 100 mM $NaHCO_3$, pH 8.0 in order to remove residual medium and fatty acids. Fatty acid methyl esters (FAMEs) were prepared from the yeast cell sediments by acidic methanolysis. For this, the cell sediments were incubated with 2 ml of 1N methanolic sulfuric acid and 2% (v/v) dimethoxypropane at 80°C for 1 h. Extraction of the FAMES was carried out by extracting twice with petroleum ether (PE). Nonderivatized fatty acids were removed by washing the organic phases in each case once with 2 ml of 100 mM $NaHCO_3$, pH 8.0 and 2 ml of distilled water. The PE phases were subsequently dried with Na_2SO_4, evaporated under argon and taken up in 100 µl of PE. The samples were separated on a DB-23 capillary column (30 m, 0.25 mm, 0.25 µm, Agilent) in a Hewlett Packard 6850 gas chromatograph with flame ionization detector. The conditions for the GLC analysis were as follows: the oven temperature was programmed from 50°C to 250°C at a rate of 5°C/min and finally at 250°C (hold) for 10 min.

The signals were identified by comparing the retention times with those of corresponding fatty acid standards (Sigma).

Acyl-CoA analysis

The acyl-CoA analysis was carried out as described in Larson and Graham (2001; Plant Journal 25: 115-125).

Expression analysis

Figures 2 A and B and figures 3 A and B depict the fatty acid profiles of transgenic C13ABYS86 yeasts fed with $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$, respectively. The substrates fed can be detected in large amounts in all transgenic yeasts. All four transgenic yeasts display synthesis of $18:3^{\Delta 6,9,12}$ and $20:3^{\Delta 8,11,14}$ and, respectively, $18:4^{\Delta 6,9,12,15}$ and $20:4^{\Delta 8,11,14,17}$, the products of the Δ-6-desaturase and Δ-6-elongase reactions, meaning that the genes PpD6 and Pse1 were able to be functionally expressed.

Figure 3 depicts, as described above, the fatty acid profiles of transgenic C13ABYS86 *S. cerevisiae* cells. The fatty acid methyl esters were synthesized by acidic methanolysis of intact cells which had been transformed either with the pESCLeu-PpD6-Pse1/pYes2 (**A**) or with the pYes2-T06E8.1/pESCLeu-PpD6-Pse1 (**B**) vectors. The yeasts were cultured in minimal medium in the presence of $18:2^{\Delta 9,12}$. The fatty acid methyl esters were subsequently analyzed by GLC.

In the control yeasts transformed with the pESCLeu-PpD6-Pse1/pYes2 vectors, the proportion of $20:3^{\Delta 8,11,14}$ to which $18:3^{\Delta 6,9,12}$ is elongated by Pse1 is substantially lower than in the yeasts which additionally express LPLAT T06E8.1. In fact, elongation of $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ was improved by 100-150% by additional expression of CeLPLAT (T06E8.1) (figure 4). This significant increase in the LCPUFA content can be

explained only as follows: the exogenously fed fatty acids ($18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$, respectively) are first incorporated into phospholipids and desaturated there by Δ-6-desaturase to give $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$. Only after reequilibration with the acyl-CoA pool can $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ be elongated by the elongase to give $20:3^{\Delta 8,11,14}$- and $20:4^{\Delta 8,11,14,17}$-CoA, respectively and then incorporated again into the lipids. LPLAT T06E8.1 is capable of converting the Δ6-desaturated acyl groups very efficiently back to CoA thioesters. Interestingly, it was also possible to improve the elongation of the fed fatty acids $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$. (Figures 2 A and B and figures 5 A and B, respectively).

Figure 5 indicates the fatty acid profiles of transgenic C13ABYS86 *S. cerevisiae* cells. Synthesis of the fatty acid methyl esters was carried out by acidic methanolysis of intact cells which had been transformed either with the vectors pESCLeu-PpD6-Pse1/pYes2 (**A**) or with the vectors pYes2-T06E8.1/pESCLeu-PpD6-Pse1 (**B**). The yeasts were cultured in minimal medium in the presence of $18:3^{\Delta 9,12,15}$. The fatty acid methyl esters were subsequently analyzed via GLC.

In contrast, expression of a different CeLPLAT (F59F4.4) has no influence on elongation (figure 4). F59F4.4 evidently does not code for an LPLAT. Thus, not every putative LPLAT nucleic acid sequence is enzymatically active in the reaction found according to the invention.

Figure 4 indicates the elongation of exogenously applied $18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$, following their endogenous Δ-6-desaturation (data of figs 2 and 5). The exogenously fed fatty acids are first incorporated into phospholipids and desaturated there to give $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$. Only after reequilibration with the acyl-CoA pool can $18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$ be elongated by the elongase to give $20:3^{\Delta 8,11,14}$- and $20:4^{\Delta 8,11,14,17}$-CoA, respectively, and then incorporated again into the lipids. LPLAT T06E8.1 is capable of converting the Δ-6-desaturated acyl groups efficiently back to CoA-thioesters.

These results show that CeLPLAT (T06E8.1), after coexpression with Δ-6-desaturase and Δ-6-elongase, leads to efficient production of C20-PUFAs. These results can be explained by the fact that CeLPLAT (T06E8.1) makes possible an efficient exchange of the newly synthesized fatty acids between lipids and the acyl-CoA pool (see figure 6).

Figure 6 indicates the acyl-CoA composition of transgenic INVSc1 yeasts transformed with the pESCLeu PpD6Pse1/pYes2 (**A**) or pESCLeu-PpD6-Pse1/pYes2-T06E8.1 (**B**) vectors. The yeast cells were cultured in minimal medium without uracil and leucine in the presence of 250 µM $18:2^{\Delta 9,12}$. The acyl-CoA derivatives were analyzed via HPLC.

When using the yeast strain INVSc1 for coexpression of CeLPLAT (T06E8.1) together with PpD6 and Pse1, the following picture emerges: control yeasts expressing PpD6 and Pse1 comprise, as already shown when using the strain C13ABYS86, only small amounts of the elongation product ($20:3^{\Delta 8,11,14}$, with 18:2 feed, and $20:4^{\Delta 8,11,14,17}$, with 18:3 feed; see figures 7 A and 8 A, respectively). Additional expression of CeLPLAT

(T06E8.1) results in a marked increase in these elongation products (see figures 7 B and 8 B). Table 5 indicates that additional expression of CeLPLAT surprisingly causes an 8-fold increase in the $20:3^{\Delta 8,11,14}$ (with 18:2 feed) and, respectively, the $20:4^{\Delta 8,11,14,17}$ (with 18:3 feed) content. It is also revealed that $C16:2^{\Delta 6,9}$ is also elongated more efficiently to give $C18:2^{\Delta 6,9}$.

The fatty acid profiles of transgenic INVSc1 S. cerevisiae cells can be seen from figure 7. Synthesis of the fatty acid methyl esters was carried out by acid methanolysis of intact cells which had been transformed either with the vectors pESCLeu-PpD6-Pse1/pYes2 (A) or pYes2-T06E8.1/pESCLeu-PpD6-Pse1 (B). The yeasts were cultured in minimal medium in the presence of $18:2^{\Delta 9,12}$. The fatty acid methyl esters were subsequently analyzed via GLC.

The fatty acid profiles of transgenic INVSc1 S. cerevisiae cells can be seen from figure 8. Synthesis of the fatty acid methyl esters was carried out by acid methanolysis of intact cells which had been transformed either with the vectors pESCLeu-PpD6-Pse1/pYes2 (**A**) or pYes2-T06E8.1/pESCLeu-PpD6-Pse1 (**B**). The yeasts were cultured in minimal medium in the presence of $18:3^{\Delta,12,15}$. The fatty acid methyl esters were subsequently analyzed via GLC.

Table 5: Fatty acid composition (in mol%) of transgenic yeasts transformed with the pESCLeu PpD6Pse1/pYes2 (PpD6 Pse1) or pESCLeu-PpD6-Pse1/pYes2-T06E8.1 (PpD6 Pse1 + T06E8) vectors. The yeast cells were cultured in minimal medium without uracil and leucine in the presence of 250 µM 18:2 $^{\Delta 9,12}$ or 18:3$^{\Delta 9,12,15}$. The fatty acid methyl esters were obtained by acidic methanolysis of whole cells and analyzed via GLC. Each value indicates the average (n = 4) ± standard deviation.

Fatty acids	Feeding with 250 µM 18:2$^{\Delta 9,12}$		Feeding with 250 µM 18:3$^{\Delta 9,12,15}$	
	PpΔ6/Pse1	PpΔ6/Pse1+ T06E8	PpΔ6/Pse1	PpΔ6/Pse1+ T06E8
16:0	15.31 ± 1.36	15.60 ± 1.36	12.20 ± 0.62	16.25 ± 1.85
16:1$^{\Delta 9}$	23.22 ± 2.16	15.80 ± 3.92	17.61 ± 1.05	14.58 ± 1.93
18:0	5.11 ± 0.63	7.98 ± 1.28	5.94 ± 0.71	7.52 ± 0.89
18:1$^{\Delta 9}$	15.09 ± 0.59	16.01 ± 2.53	15.62 ± 0.34	15.14 ± 2.61
18:1$^{\Delta 11}$	4.64 ± 1.09	11.80 ± 1.12	4.56 ± 0.18	13.07 ± 1.66
18:2$^{\Delta 9,12}$	28.72 ± 3.25	14.44 ± 1.61	-	-
18:3$^{\Delta 6,9,12}$	3.77 ± 0.41	4.72 ± 0.72	-	-
18:3$^{\Delta 9,12,15}$	-	-	32.86 ± 1.20	14.14 ± 2.52
18:4$^{\Delta 6,9,12,15}$	-	-	5.16 ± 1.04	3.31 ± 1.15
20:2$^{\Delta 11,14}$	2.12 ± 0.86	4.95 ± 4.71	-	-
20:3$^{\Delta 8,11,14}$	1.03 ± 0.14	8.23 ± 1.59	-	-
20:3$^{\Delta 11,14,17}$	-	-	4.12 ± 1.54	6.95 ± 2.52
20:4$^{\Delta 8,11,14,17}$	-	-	1.34 ± 0.28	8.70 ± 1.11

A measure for the efficiency of LCPUFA biosynthesis in transgenic yeast is the quotient of the content of the desired Δ-6-elongation product after Δ-6-desaturation (20:3$^{\Delta 6,11,14}$ and 20:4$^{\Delta 8,11,14,17}$, respectively) to the content of fatty acid fed in (18:2$^{\Delta 9,12}$ and 18:3$^{\Delta 9,12,15}$, respectively). This quotient is 0.04 in INVSc1 control yeasts expressing PpD6 and Pse1, and 0.60 in yeasts expressing CeLPLAT in addition to PpD6 and

Pse1. In other words: the content of desired Δ-6-elongation product after Δ-6-desaturation with coexpression of CeLPLAT is 60% of the content of the fatty acid fed in in each case. In control yeasts, this content is only approx. 4%, meaning a 15-fold increase in the efficiency of LCPUFA biosynthesis in transgenic yeast due to coexpression of LPLAT.

Interestingly, coexpression of CeLPLAT causes not only an increase in the elongation products mentioned, $20:3^{\Delta 8,11,14}$ and $20:4^{\Delta 8,11,14,17}$, but also an increase in the $20:3^{\Delta 8,11,14}$: $20:2^{\Delta 11,14}$ ratio and the $20:4^{\Delta 8,11,14,17}$: $20:3^{\Delta 11,14,17}$ ratio, respectively. This means that, in the presence of LPLAT, Δ-6-elongase preferably uses polyunsaturated fatty acids ($18:3^{\Delta 6,9,12}$ and $18:4^{\Delta 6,9,12,15}$) as substrate, while no distinct substrate specificity is discernible in the absence of LPLAT ($18:2^{\Delta 9,12}$ and $18:3^{\Delta 9,12,15}$ are also elongated). The reason for this may be protein-protein interactions between Δ-6-elongase, Δ-6-desaturase and LPLAT or posttranslational modifications (partial proteolysis, for example). This will also explain why the above-described rise in Δ-6-elongation products with coexpression of Δ-6-desaturase, Δ-6-elongase and LPLAT is smaller when a protease-deficient yeast strain is used.

Acyl-CoA analyses of transgenic INVSc1 yeasts fed with $18:2^{\Delta 9,12}$ gave the following result: no $18:3^{\Delta 6,9,12}$-CoA and $20:3^{\Delta 8,11,14}$-CoA is detectable in control yeasts expressing PpD6 and Pse1, indicating that neither the substrate ($18:3^{\Delta 6,9,12}$-CoA) nor the product ($20:3^{\Delta 8,11,14}$-CoA) of Δ-6-elongase is present in detectable amounts in control yeasts. This suggests that the transfer of $18:3^{\Delta 6,9,12}$ from membrane lipids into the acyl-CoA pool does not take place or does not take place correctly, meaning that there is hardly any substrate available for the Δ-6-elongase present, and this in turn explains the low elongation product content in control yeasts. INVSc1 yeasts which express CeLPLAT in addition to PpD6 and Pse1 and which had been fed with $18:2^{\Delta 9,12}$ have substantial amounts of $20:3^{\Delta 8,11,14}$-CoA but not of $18:3^{\Delta 6,9,12}$-CoA. This indicates that LPLAT transfers $18:3^{\Delta 6,9,12}$ from the membrane lipids to the acyl-CoA pool very efficiently. $18:3^{\Delta 6,9,12}$-CoA is then elongated by Δ-6-elongase so that $20:3^{\Delta 8,11,14}$-CoA but not any $18:3^{\Delta 6,9,12}$-CoA is detectable.

b) Functional characterization of the CeLPLATs in transgenic plants

Expression of functional CeLPLAT in transgenic plants

DE 102 19 203 describes transgenic plants whose seed oil comprises small amounts of ARA and EPA, due to seed-specific expression of functional genes coding for Δ-6-desaturase, Δ-6-elongase and Δ-5-desaturase. The vector exploited for transformation of these plants can be found in SEQ ID NO: 56. In order to increase the content of these LCPUFAs, the gene CeLPLAT (T06E8.1) was additionally expressed in seeds in the transgenic plants mentioned.

For this purpose, the coding region of CeLPLAT was amplified via PCR.

Table 6 indicates the primers used for cloning another ceLPLAT clone into binary vectors.

Table 6: Nucleotide sequences of the PCR primers for cloning CeLPLAT (T06E8.1) into the binary vector pSUN3

Primer	Nucleotide sequence
ARe503f*	5' TTAAGCGCGGCCGCATGGAGAACTTCTGGTCG 3'
ARe504r*	5' ACCTCGGCGGCCGCCCTTTTACTCAGATTTC 3'

* f: forward, r: reverse

The PCR product was cloned into a pENTRY vector between USP promoter and OCS terminator. The expression cassette was then cloned into the binary pSUN300 vectors. The vector obtained was referred to as pSUN3CeLPLAT (figure 1). In addition, the CeLPLAT coding regions were amplified and cloned between LegB4 promoter and OCS terminator. This vector was referred to as pGPTVCeLPLAT (figure 9A).

In addition, the CeLPLAT coding region was amplified via PCR and cloned between LegB4 promoter and OCS terminator. The PCR primers used for this were selected so as for an efficient Kosak sequence to be introduced into the PCR product. Moreover, the CeLPLAT DNA sequence was modified so as to adapt it to the codon usage of higher plants.

The following primers were used for the PCR:

Forward primer: 5'-ACATAATGGAGAACTTCTGGTCTATTGTTGTGTTTTTTCTA-3'

Reverse primer: 5'- CTAGCTAGCTTACTCAGATTTCTTCCCGTCTTTTGTTTCTC-3'

The PCR product was cloned into the cloning vector pCR Script and cloned via the restriction enzymes XmaI and SacI into the vector pGPTV LegB4-700. The resulting plasmid was referred to as pGPTV LegB4-700 + T06E8.1 (figure 9A).

The same PCR product was in addition cloned into a multi-gene expression vector which already comprised the genes for a Phaeodactylum tricornutum delta-6-desaturase (SEQ ID NO: 69, amino acid sequence SEQ ID NO: 70) and a P. patens delta-6-elongase. The resulting plasmid was referred to as pGPTV USP/OCS-1,2,3 PSE1(Pp)+D6-Des(Pt)+2AT (T06E8-1) (figure 9B). The sequences of the vector and of the genes can be found in SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73 and SEQ ID NO: 74. The Phaeodactylum tricornutum Δ-6-desaturase extends from nucleotide 4554 to 5987 in SEQ ID NO: 71. The Physcomitrella patens Δ-6-elongase extends from nucleotide 1026 to 1898 and that of Caenorhabditis elegans LPLAT extends from

nucleotide 2805 to 3653 in SEQ ID NO: 71.

Tobacco plants were cotransformed with the pSUN3CeLPLAT vector and the vector described in DE 102 19 203 and SEQ ID NO: 56, which comprises genes coding for Δ-6-desaturase, Δ-6-elongase and Δ-5-desaturase, with transgenic plants being selected using kanamycin.

Tobacco plants were moreover transformed with the pGPTV USP/OCS-1,2,3 PSE1(Pp)+D6-Des(Pt)+2AT (T06E8-1) vector [see SEQ ID NO: 71, SEQ ID NO: 72, SEQ ID NO: 73 and SEQ ID NO: 74].

Linseed was transformed with the pSUN3CeLPLAT vector. The resulting transgenic plants were crossed with those transgenic linseed plants which already comprised small amounts of ARA and EPA, owing to functional gene expression of Δ-6-desaturase, Δ-6-elongase and Δ-5-desaturase.

Linseed was furthermore transformed with the pGPTV LegB4-700 + T06E8.1 vector. The resulting transgenic plants were crossed with those transgenic linseed plants which already comprised small amounts of ARA and EPA, owing to functional expression of Δ-6-desaturase, Δ-6-elongase and Δ-5-desaturase.

The seeds of transgenic tobacco and linseed plants were, as described hereinbefore [example 3 b)], studied for increased LCPUFA contents.

The function of acyl-CoA:lysophospholipid acyltransferase (LPLAT) can be deduced from the studies presented herein as depicted in figure 10 A and 10 B. The biosynthetic pathway of LCPUFAS is thus as follows.

Desaturases catalyze the introduction of double bonds into lipid-coupled fatty acids (*sn2*-acyl-phosphatidylcholine), while the elongases exclusively catalyze the elongation of coenzyme A-esterified fatty acids (acyl-CoAs). According to this mechanism, the alternating action of desaturases and elongases requires continuous exchange of acyl substrates between phospholipids and acyl-CoA pool and thus the existence of an additional activity which converts the acyl substrates to the substrate form required in each case, i.e. lipids (for desaturases) or CoA thioesters (for elongases). This exchange between acyl-CoA pool and phospholipids is made possible by LCPUFA-specific LPLAT. The biosynthesis of ARA (**A**) takes place analogously to that of EPA (**B**), but with the difference that, in the case of EPA, a Δ-15-desaturation takes place upstream of the Δ-6-desaturation so that α18:3-PC acts as a substrate for Δ-6-desaturase. The biosynthesis of DHA requires a further exchange between phospholipids and acyl-CoA pool via LPLAT: $20{:}5^{\Delta 5,8,11,14,17}$ is transferred from the phospholipids pool to the CoA pool and, after Δ-5-elongation, $22{:}5^{\Delta 7,10,13,16,19}$ is transferred from the CoA pool to the phospholipids pool and finally converted by Δ-4-desaturase to give DHA. The same applies to the exchange in the biosynthetic pathway using Δ-8-desaturase, Δ-9-elongase and Δ-5-desaturase.

Example 5: Functional characterization of the acyltransferases

To compare the substrate specificity of acyltransferases of higher plants and LCPUFA-producing organisms, microsomal fractions were isolated from the LCPUFA-producing organism Mortierella alpina and from sunflower. The GPAT and LPAAT activities were assayed with different acyl-CoAs as substrate.

A position analysis of the lipids was carried out to verify whether the LCPUFA producer Thraustochytrium does indeed incorporate DHA at the sn-2 position of the lipids.

To isolate LCPUFA-specific acyltransferases, cDNA libraries were established starting from mRNA of the LCPUFA-producing organisms Thraustochytrium, Physcomitrella, Cryptecodinium cohnii and Fusarium and a Shewanella genomic library was established, and these libraries were analyzed in greater detail via DNA sequencing. Acyltransferase clones were identified via sequence homologies. As an alternative, acyltransferases were amplified via PCR techniques.

Transgenic E. coli cells, yeasts, insect cells and plant cells with an elevated expression of at least one LCPUFA-specific acyltransferase have an elevated LCPUFA content in their lipids.

Example 6: Isolation of microsomal fractions from Mortierella, sunflower and linseed, and analysis of the substrate specificity of acyltransferases for different acyl-CoAs.

To find out whether higher plants, in particular oil seed plants such as sunflower, linseed, oilseed rape or soybean, can incorporate LCPUFAs into their lipids, microsomes were prepared from sunflower and linseed, and different acyltransferase activities were studied for their substrate specificity for LCPUFA-CoAs. Specifically, GPAT, LPAAT and LPCAT activities were studied. These results were compared with the corresponding acyltransferase activities of the LCPUFA producers Mortierella alpina, which, as is known, comprises high levels of the LCPUFA arachidonic acid in its lipids and in the triacylglycerol (C. Ming et al. (1999) Bioresource Technology 67: 101-110).

Preparation of microsomal membranes from cotyledons of maturing seeds of sunflower and linseed

All the procedures were carried out at 4°C. The cotyledons of maturing sunflower seeds and linseed were harvested approximately 10 days after anthesis and suspended in 0.1 M sodium phosphate buffer (pH 7.2), comprising 0.33 M sucrose and 0.1% BSA (free from fatty acids). After comminution in a glass homogenizer, the homogenate was centrifuged for 30 minutes at 20 000 x g. The supernatant was filtered through one layer of Miracloth and centrifuged for 90 minutes in an ultracentrifuge at 100 000 x g. The pelleted microsomal membranes were washed with 0.1 M sodium phosphate buffer (pH 7.2) and resuspended in a small volume of buffer,

using a glass homogenizer. The microsomal membrane preparations were either immediately processed or stored at -80°C.

Preparation of microsomal membranes from Mortierella

Mortierella cultures were harvested after 5 days and placed on ice. All further procedures were carried out at 4°C. The mycelium was suspended in 0.1 M sodium phosphate buffer (pH 7.2), comprising 0.33 M sucrose, 0.1% BSA (free from fatty acids), 1000 units of catalase/ml and 1 mM Pefabloc. The following steps were carried out as described under "preparations of microsomal membranes from cotyledons of maturing seeds of sunflower and linseed".

Acyl-CoA substrate specificity of GPAT: conversion of individual acyl-CoA substrates in the acylation of [^{14}C] glycerol-3-phosphate

The specificity of the GPAT was studied to verify whether the enzyme has a preference for certain acyl-CoAs, in particular to determine whether the GPAT from oil seed plants converts LCPUFA-CoAs. Microsomal membranes were incubated with 0.5 mM (Mortierella) or 0.2 mM (sunflower and linseed) of one of the following acyl-CoAs: myristoyl-CoA (14:0-CoA), palmitoyl-CoA (16:0-CoA), palmitoleoyl-CoA (16:1-CoA), stearoyl-CoA (18:0-CoA), oleoyl-CoA (18:1-CoA), linoleoyl-CoA (18:2-CoA), dihomo-gamma-linolenoyl-CoA (20:3-CoA) or arachidonyl-CoA (20:4-CoA) and 5 mM [^{14}C] G3P. Microsomal membranes (equivalent to 50 µg of protein in the case of sunflower and Mortierella and 150 µg of protein in the case of linseed) were added to the reaction mixture in order to start the reaction. After incubation for 5 minutes, the lipids were extracted by the method of Bligh & Dyer, and the radioactivity incorporated in complex lipids was determined.

Figure 11 and table 7a and 7b show the GPAT activities of Mortierella, sunflower and linseed for different acyl-CoA substrates.

The GPAT of Mortierella incorporates unsaturated fatty acids more efficiently than saturated fatty acids. Oleate and linoleate were converted with similar incorporation rates (100% and 90%, respectively). The incorporation of polyunsaturated fatty acids (20:3-CoA and 20:4-CoA) was only marginally lower (80% and 75%, respectively).

Oleate and linoleate are also the best substrates for GPAT in microsomal membranes (100% and 85% activity, respectively). Acyl-CoAs of the saturated fatty acids stearate and palmitate are only incorporated approximately half as efficiently (40% and 64%, respectively). This also applies analogously for 20:3-CoA (55%). Arachidonyl-CoA is a relatively poor substrate for sunflower GPAT (23%).

The GPAT in microsomal membranes of linseed has the lowest specific activity of all GPAT enzymes studied. With 6 nmol/min/mg protein, it is only half as active as sunflower GPAT and 5 times less active than the enzyme from Mortierella. As regards the substrate specificities behaves The most efficient acyl-CoA substrates of the

linseed GPAT are oleate and linoleate (100% and 90%, respectively), as is the case with sunflower. The incorporation rates of the saturated fatty acids stearate and palmitate, at 65% and 90%, are markedly higher than in the case of sunflower. In contrast, arachidonyl-CoA is a very poor substrate for linseed GPAT (5%).

Acyl-CoA substrate specificity of LPAAT: conversion of individual acyl-CoA substrates in the acylation of lysophosphatidic acid

The specificity of the LPAAT was studied in order to verify whether the enzyme has a preference for certain acyl-CoAs, in particular to determine whether the LPAAT from oil seed plants converts LCPUFA-CoAs. LPAAT activity was determined in a continuous spectraphotometric assay in which 5,5-dithiobis-2-nitrobenzoate (DTNB) was used, and the change in absorption at 409 nm and 25°C was monitored (F.M. Jackson et al. (1998) Microbiology 144: 2639-2645). The assay comprised sn-1-oleoyl-lysophosphatidic acid (30 nmol), DTNB (50 nmol) and 20 nmol of one of the following acyl-CoAs: palmitoyl-CoA (16:0-CoA), stearoyl-CoA (18:0-CoA), oleoyl-CoA (18:1-CoA), linoleoyl-CoA (18:2-CoA), dihomo-gamma-linolenyl-CoA (20:3-CoA) or arachidonyl-CoA (20:4-CoA) in 1 ml of 0.1 M phosphate buffer, pH 7.2. The CoA liberated in the reaction was determined quantitatively with the aid of the initial increase and the absorption coefficient of 13.6 mM-1 x cm-1. Microsomal membranes (equivalent to 10 µg of protein in the case of Mortierella and 40 µg of protein in the case of sunflower and linseed) were added to the reaction mixture in order to start the reaction.

Figure 11 and table 7a and 7b show the LPAAT activities of Mortierella, sunflower and linseed for different acyl-CoA substrates.

The Mortierella LPAAT incorporates oleoyl-CoA most efficiently (100%). Linoleoyl-CoA is likewise converted very efficiently (90%). While the saturated fatty acid substrates 16:0-CoA and 18:0-CoA are only incorporated at 40% and 36%, respectively, the LCPUFA substrates 20:3-CoA and 20:4-CoA are incorporated with a relatively high efficiency (in each case 65%).

In sunflower microsomal membranes, linoleoyl-CoA is the LPAAT substrate which is most efficiently incorporated into phosphatidic acid (250% relative to oleoyl-CoA). Both saturated and polyunsaturated acyl-CoA were poor substrates for sunflower LPAAT (relative activities less than 20%).

A very similar picture emerges for linseed LPAAT: linoleoyl-CoA is the best substrate (120% relative to oleoyl-CoA). Saturated fatty acids are poor LPAAT substrates (25% and 30% for 16:0-CoA and 18:0-CoA). Arachidonyl-CoA is converted least (19% relative activity).

Acyl-CoA substrate specificity of LPCAT: conversion of individual acyl-CoA substrates in the acylation of lysophosphatidylcholine

In higher plants and fungi, fatty acids are desaturated for the production of polyunsaturated fatty acids while esterified with phosphatidylcholine (PC) (A.K. Stobart and S. Stymne (1985) Planta 163: 119-125; F.M. Jackson et al. (1998) Microbiology 144: 2639-2645). The involvement of PC in the desaturation of fatty acids also in fungi requires the existence of a functional transfer system for fatty acids into and from the sn-2 position of PC, analogously to the system which has been described for developing oil seeds (Jackson et al., 1998; Stobart et al., 1983). It is assumed that this transfer of the acyl group from acyl-CoA to the sn-2 position of PC is catalyzed by LPCAT. In the present context, the specificity of LPCAT was studied in order to verify whether the enzyme has a preference for certain acyl-CoAs, in particular in order to determine whether the oil seed LPCAT converts LCPUFA-CoAs.

LPCAT activity was determined in a continuous spectraphotometric assay in which 5,5-dithiobis-2-nitrobenzoate (DTNB) was used, and the change in absorption at 409 nm and 25°C was monitored. The assay comprised sn-1-palmitolysophosphatidylcholine (30 nmol) as acyl acceptor, DTNB (50 nmol) and 20 nmol of one of the following acyl-CoAs: myristoyl-CoA (14:0-CoA), palmitoyl-CoA (16:0-CoA), palmitoleoyl-CoA (16:1-CoA), stearoyl-CoA (18:0-CoA), oleoyl-CoA (18:1-CoA), linoleoyl-CoA (18:2-CoA), dihomo-gamma-linolenyl-CcA (20:3-CoA) or arachidonyl-CoA (20:4-CoA) in 1 ml of 0.1 M phosphate buffer, pH 7.2. The reaction was started by addition of microsomal membrane preparation. The amount of microsomal membrane preparation added was 5 µg (Mortierella and sunflower) or 30 µg (linseed). The CoA liberated in the reaction was determined quantitatively with the aid of the initial increase and the absorption coefficient of 13.6 mM-1 x cm-1 at 409 nm.

Figure 12 and table 7a and 7b show the LPCAT activities of Mortierella, sunflower and linseed for different acyl-CoA substrates.

The results demonstrate that LPCAT is considerably more active in microsomal membranes of sunflower and Mortierella than in the case of linseed (see tables 10a and 10b). Besides 18:1 (100%), Mortierella LPCAT also converts 18:2 (40%), 20:3 (85%) and 20:4 (90%) with high efficiency. Saturated fatty acids are virtually not converted (relative activity less than 25%).

Sunflower LPCAT converts oleoyl-CoA and linoleoyl-CoA with similar efficiency (100% and 120% relative activities, respectively). Palmitoyl-CoA and stearoyl-CoA are poor substrates (relative activity less than 20%). 20:3-CoA and 20:4-CoA are virtually not converted (relative activities less than 5%).

The behavior of linseed LPCAT is similar: while oleoyl-CoA and linoleoyl-CoA are converted equally efficiently, no LPCAT activity was detected for 20:3-CoA and 20:4-CoA.

Discussion of the data for the acyl-CoA specificity of GPAT, LPAAT and LPCAT

The substrate specificity of G3P-acylating enzymes was studied intensively in order to understand the mechanism of the distribution of fatty acids in phospholipids and triacylglycerol. Mammalian microsomal GPAT utilizes saturated and unsaturated acyl-CoAs (Yamada & Okuyama, 1978; Haldar et al., 1979; Tamai & Lands, 1974). The same was demonstrated for plant microsomal GPATs (Frentzen, 1993; Bafor et al. 1990). Jackson et al. (1998) furthermore demonstrated that neither GPAT nor LPAAT from the fungus Mucor circinelloides has a pronounced substrate specificity for acyl-CoAs. In the case of Mucor, both saturated and unsaturated fatty acids are acylated at both positions. A purified GPAT from the membrane fraction of Mortierella ramanniana, in contrast, showed a clear preference for oleoyl-CoA in contrast to palmitoyl-CoA (Mishra & Kamisaka, 2001).

In order to study whether GPAT in microsomal membranes from Mortierella, sunflower and linseed has a pronounced specificity for certain acyl-CoA species, individual acyl-CoAs were added to the microsomes. The Mortierella GPAT has a similarity with other plant, animal and fungal GPATs in as far as it has a broad specificity for acyl-CoAs, i.e. saturated and unsaturated fatty acids are acylated at the sn-1 position of G3P. The GPATs from sunflower and linseed microsomal membranes also utilize saturated and unsaturated acyl donors in a manner similar to what has been demonstrated for safflower and turnip rape (Bafor et al., 1990), albeit with a preference for unsaturated fatty acids. In general, the Mortierella GPAT is less discriminating than the sunflower and linseed enzyme. However, it is noticeable that sunflower and linseed GPATs virtually fails to convert arachidonyl-CoA, whereas the Mortierella enzyme acylates arachidonyl-CoA in a highly efficient manner.

In the second acylation step, Mortierella, sunflower and linseed LPAAT is active with sn-1-oleoyl lysophosphatidic acid as acyl acceptor. Similarly to GPAT, Mortierella LPAAT also has a broad specificity for acyl-CoAs. These data resemble those from guinea pig and rat liver microsomes, where, with the exception of stearoyl-CoA, LPAAT esterifies all acyl-CoAs with 16 and 18 carbon atoms, independently of the degree of saturation (Hill and Lands, 1968). In the present work, the sunflower and linseed LPAATs showed a pronounced specificity for linoleate and oleate. Saturated fatty acids, in contrast, were scarcely converted. These data agree with the observation that, in most oil seed crops, LPAATs show a higher specificity for unsaturated fatty acids (Griffiths et al., 1985; Ichihara et al., 1987). In sunflower and linseed, arachidonyl-CoA is a poor substrate, even for LPAAT. In comparison with GPAT, the LPAAT activity of sunflower and linseed is somewhat higher, however.

The specificity of LPCAT in microsomal preparations of Mortierella and sunflower was likewise studied. In Mortierella, LPCAT showed a broad spectrum of substrate specificity. The activity of the enzyme with different acyl-CoAs decreased in the order 18:1-CoA > 20:4-CoA > 20:3-CoA > 16:1-CoA > 18:2-CoA. Sunflower and linseed LPCAT showed virtually no activity with 20:3 and 20:4-CoA. LPCAT in bovine brain microsomes also showed a weak activity with saturated acyl-CoAs and a more pronounced activity with linoleoyl- and oleoyl-CoA (Deka et al., 1986). LPCAT from

bovine heart muscle microsomes accept a wide range of substrates, although the activity is particularly high with arachidonyl-, linoleoyl- and oleoyl-CoA substrates (Sanjawara et al., 1988). In plants, the acyl specificity and selectivity of LPCAT was studied in microsomes of safflower (Stymne et al., 1983; Griffith et al., 1985) and linseed (Stymne & Stobart, 1985a). Oleate and linoleate were acylated with approximately the same conversion rate at the sn-2 position of PC. The activity with alpha-linoleate was only approximately half as much. Palmitate and stearate were considerably poorer LPCAT substrates when they were offered as individual acyl-CoAs. If a mixture of saturated and unsaturated acyl-CoAs was offered, palmitate and stearate were completely excluded by the PC. LPCAT in microsomal membranes of Mucor circinelloides too utilizes oleoyl- and linoleoyl-CoA much more efficiently than saturated fatty acids. There is thus a great degree of agreement in the specificity of plant, animal and fungal LPCATs. The fact that LPCAT from Mortierella microsomal membranes only shows poor activity with stearoyl-CoA and good activity with oleoyl- and linoleoyl-CoA might suggest that phosphatidylcholine acts as substrate for desaturases. It was demonstrated that oleate at the sn-1 and the sn-2 position of PC acts as substrate for Δ-12-desaturase in oil seed plants (Stymne & Stobart, 1986; Griffiths et al., 1988). Similar results were reported for Mucor circinelloides (Jackson et al., 1998). Δ-6-Desaturase also utilizes linoleate at the sn-2 position of PC in microsomal membrane preparations of Mucor (Jackson et al., 1998). The Δ-6-desaturase from borage, too, utilizes exclusively linoleate at the sn-2 position of the phospholipid (Stymne & Stobart, 1986; Griffiths et al., 1988).

The results described in example 6 demonstrate that acyltransferases from sunflower and linseed are not capable of efficiently incorporating LCPUFAs such as dihomo-γ-linolenate and arachidonate into the membrane and storage lipids. While LCPUFAs can be produced in oil seed plants such as sunflower, linseed or soybean, by functionally expressing the biosynthetic genes in question, it can be assumed that the resulting LCPUFAs are not efficiently incorporated into triacylglycerol as the result of lacking acyltransferase activities, which leads to a poor yield. Thus, acyltransferases with a high specificity for LCPUFA-CoAs must be transformed into oil seed plants in addition to LCPUFA biosynthetic genes (for example desaturases and elongases or polyketide synthases). Suitable for this purpose are acyltransferases from LCPUFA-producing organisms such as Mortierella, Phaeodactylum, Crypthecodinium, Physcomitrella, Euglena and Thraustochytrium.

Table 7a and 7b indicate the activity and acyl specificity of linseed, sunflower and Mortierella alpina acyltransferases.

Table 7a: Activity and acyl specificity of linseed and sunflower acyltransferases

Enzyme activity	**Linseed**			**Sunflower**		
	GPAT	LPAAT	LPCAT	GPAT	LPAAT	LPCAT
Rate (nmol/min/mg protein) of the incorporation of oleic acid	6	25	9	13	28	360
Percentage incorporation in comparison with the incorporation of oleic acid						
Myristoyl-CoA	100	30	0	57	16	1
SSA Palmitoyl-CoA	90	25	5	64	15	13
Palmitololeoyl-CoA		140	180		140	90
Stearoyl-CoA	65	30	15	40	14	18
Oleoyl-CoA	100	100	100	100	100	100
Linoleoyl-CoA	90	120	100	85	250	120
20:3-CoA			0	55		3
Arachidonoyl-CoA	5	19	0	23	18	4

Table 7b: Activity and acyl specificity of Mortierella alpina acyltransferases

Enzyme activity	*Mortierella alpina*		
	GPAT	LPAAT	LPCAT
Rate (nmol/min/mg protein) of the incorporation of oleic acid	30	51	350
Percentage incorporation in comparison with the incorporation of oleic acid			
Myristoyl-CoA		55	0
Palmitoyl-CoA	66	40	25
Palmitololeoyl-CoA		70	60
Stearoyl-CoA	50	36	10
Oleoyl-CoA	100	100	100
Linoleoyl-CoA	90	90	40
20:3-CoA	80	65	85
Arachidonoyl-CoA	75	65	90

Example 7: Position analysis of the lipids from Thraustochytrium

It was demonstrated in example 6 that LCPUFA producers such as Mortierella have membrane-bound acyltransferase activities which incorporate LCPUFA-CoAs into membrane and storage lipids. Position analyses of the lipids from LCPUFA producers allow conclusions to be drawn regarding the in-vivo activities of the individual acyltransferases. This is why the question of which fatty acids are esterified at the individual positions of the lipids of the DHA producer Thraustochytrium was studied herein below.

a) Cultivation of Thraustochytrium spec.(TS) ATCC 26185

Cultivation of the fungus TS was performed in TS liquid culture and by streaking onto TS plates. Every three weeks, the fungi were transferred to fresh plates, stored for two days at 28°C and thereafter stored at RT (approx. 23°C). The liquid culture was incubated with shaking at 30°C and harvested after 6 days. Shaking the culture with exposure to light increases the lipid yield (data not shown).

l) TS medium: (Bajpai et al. (1991) JAOCS 68: 507-514)

a) 10x solution A (g/l):

250 g/l	NaCl
50 g/l	$MgSO_4 7H_2O$
10 g/l	KCl
20 g/l	Na glutamate
2 g/l	$(NH4)_2SO_4$
20 g/l	glucose

Autoclave solution.

b) 10x solution B (g/l)

200 g/l	glucose
20 g/l	yeast extract

Solution B was filter-sterilized.

c) 10x solution C (g/l)

2 g/l	$CaCO_3$

To dissolve the $CaCO_3$, the solution was acidified with HCl and thereafter autoclaved.

d) 10x solution D (g/l)

1 g/l	KH_2PO_4
1 g/l	$NaHCO_3$

The solution was autoclaved.

Supplements: thiamine and vitamin B_{12}

In each case 100 ml of the 10× solutions a) to d) and 10 µg/l thiamine and 1 µg/l vitamin B_{12} were added to 600 ml of autoclaved distilled water.

b) Lipid analysis of Thraustochytrium (Bligh & Dyer (1959) Canadian J. Biochem. 37: 911-917)

To extract the total lipids from TS in liquid culture, the former were sedimented by centrifugation for 10 minutes at 3000 g. Resuspension of the cells in 10 ml of 0.45% NaCl was followed by boiling for 10 minutes in a water bath. After a further centrifugation step (as above) of the suspension, which had been transferred into 40 ml ground-glass tubes, the sediment was taken up in trichloromethane/methanol 1:2 (v/v). Here, the volume of the solvent mixture depended on the volume of the sediment. In general, 10 ml of the mixture were required for extracting a 100 ml culture. The first

extraction took place for at least 6 hours, but mostly overnight at 8°C on a shaker. Thereafter, what remained of the cells was resedimented and the supernatant was stored at 8°C. The second extraction was performed analogously to the first extraction, however using trichloromethane/methanol 2:1 (v/v) overnight. After the second extraction, what was left of the cells was resedimented, and the supernatant was combined with that of the first extraction. Then, the combined extracts were brought to a trichloromethane/methanol/0.45% NaCl ratio of 2:1:0.7 and shaken. Here, undesired coextracted substances such as sugars are extracted by shaking and then enter aqueous phase. Then, the extract was centrifuged until phase separation occurred, the organic bottom phase was removed and filtered through cotton wool into a round-bottomed flask to remove suspended matter. The lipid extract was evaporated to dryness on a rotary evaporator, the total lipids were transferred into trichloromethane/methanol 2:1 (v/v) and into a ground-glass tube. Then, the extract was again evaporated to dryness under nitrogen and finally taken up in trichloromethane/methanol 2:1 (v/v) in a defined volume.

c) Lipid analysis from Thraustochytrium membranes

Isolated Thraustochytrium membranes were transferred into a ground-glass tube, taken up in 0.45% NaCl and boiled for 5 minutes in a water bath to inactivate lipid-degrading enzymes. After centrifugation (5 minutes, 3000 x g), the aqueous supernatant was decanted off. The lipids were extracted for one hour at 4°C in trichloromethane/methanol (2:1). After addition of 1/3 volume of 0.45% NaCl, the samples were centrifuged to improve phase separation (5 minutes, 3000 x g). The lipid-containing bottom phase was removed and concentrated in vacuo. The lipids were taken up in a suitable volume of trichloromethane.

Directly thereafter, the lipids were applied to silica gel plates (silica gel 60, 20 x 20 cm, 0.25 mm layer thickness; Merck, Darmstadt) for subjecting the phospholipids to thin-layer chromatographic separation, together with suitable standards. The mobile phase used was trichloromethane/methanol/glacial acetic acid/H_2O 91/30/4/4 (v/v/v/v). The development time was 1.5 hours. After the solvent had been evaporated, the plates were stained with 2′,7′-dichlorofluorescein (Merck, Darmstadt; in 0.3% isopropanol) and visualized under UV light (366 nm).

d) Lipase digestion of the Thraustochytrium total lipids

The enzymatic digestion is performed by means of pancreatic lipase (EC 3.1.1.3). The hydrolytic cleavage takes place at the phase boundary between fat and water, the enzyme specifically attacking the terminal ester bonds in the *sn*-1 and *sn*-3 positions in triacylglycerols (TAGs). An intermediary concentration of 1,2- and 2,3-diacyl-*sn*-glycerols, which are subsequently digested further to give *sn*-2 monoacylglycerols, takes place. Following separation by thin-layer chromatography and recovery of the sn-2 monoacylglycerol fraction, the fatty acid composition of the TAGs in the middle position is determined.

50 mg of the total lipid were weighed into a ground-glass tube. After addition of 0.5 ml of Tris buffer, 0.1 ml of $CaCl_2$ solution and 0.25 ml of bile salt solution (0.05% (w/v) bile salt; Sigma, Deisenhofen), the ground tube was sealed. The mixture was mixed for one minute and subsequently prewarmed for one minute in a water bath at 40°C in order to emulsify the sample.

Hydrolysis was effected after addition of pancreatic lipase (EC 3.1.1.3; Sigma, Deisenhofen; 2 mg of lipase per 5 mg of lipid; lipase freshly dissolved in 0.5 ml of Tris buffer) at 38°C and high shaking frequency (if possible 1200 rpm). After 30 minutes, the reaction was stopped by addition of 1 ml of HCl (6 N) and 1 ml of ethanol.

The reaction mixture was extracted twice in the centrifuge glass, using in each case 4 ml of diethyl ether. In doing so, the ether phase, which was the top phase, was removed. The aqueous phase which remained was reextracted with diethyl ether. The formation of emulsions was additionally prevented in each extraction step by centrifugation. The combined ether phases were washed by shaking with in each case 3 ml of water (distilled). The organic phase was transferred into a fresh tube and dried using sodium sulfate. After centrifugation for 2 minutes at 3000 x g, the clear supernatant was removed and the sodium sulfate pellet was again extracted by shaking with diethyl ether, centrifuged as stated above, and the organic phases were combined. After concentration of the ether extract in vacuo, the extract was immediately thereafter applied to silica gel plates (silica gel 60, 20 x 20 cm, 0.25 mm layer thickness; Merck, Darmstadt) in order to subject the partial glycerides to separation by thin-layer chromatography. The mobile phase used was diisopropyl ether/glacial acetic acid 40:1 (v/v). The development time was 35-45 minutes. After evaporation of the solvent, the plates were stained using 2′,7′-dichlorofluorescein (Merck, Darmstadt; in 0.3% isopropanol) and visualized under UV light. The individual lipid fractions were separated in the following order: monoacylglycerols (*sn*-2 MAGs, immediately above the starting line), diacylglycerols (*sn*-1,2- and *sn*-2,3-DAGs), free fatty acids (FFA) and the unreacted TAGs.

The MAG band was scraped off from the silica gel plate. The fatty acid composition of the TAGs was determined by means of transmethylation, followed by gas-chromatographic separation of the fatty acid methyl esters (FAMEs).

Tris buffer:

1M Tris/HCl, bring to pH 8.0 using HCl

CaCl solution

2.2% (w/v) $CaCl_2$

e) Lipase digestion of the Thraustochytrium membrane lipids (Fischer et al., 1973)

The position analysis of the membrane lipids was carried out by enzymatic hydrolysis of the sn-2 ester bond with phospholipase A_2 (EC 3.1.1.4).

The isolated membrane lipids were concentrated in vacuo, treated with 0.5 ml of hydrolysis buffer and dispersed for 5 minutes using a sonicator. Hydrolysis was effected at RT after addition of 50 U of phospholipase A_2. The reaction was stopped by addition of 4 ml of trichloromethane/methanol 2:1 (v/v) and 0.45% NaCl. The organic, bottom phase was transferred into a fresh vessel, evaporated on a rotary evaporator and taken up in 200 µl of trichloromethane/methanol 2:1 (v/v).

Directly thereafter, the mixture was applied to silica gel plates (silica gel 60, 20 x 20 cm, 0.25 mm layer thickness; Merck, Darmstadt) in order to subject the phospholipids to thin-layer chromatographic separation. The mobile phase used was trichloromethane/methanol/glacial acetic acid/H_2O 91/30/4/4 (v/v/v/v). The development time was 1.5 hours. After evaporation of the solvent, the plates were stained using 2′,7′-dichlorofluorescein (Merck, Darmstadt; in 0.3% isopropanol) and visualized under UV light. Bands of interest were scraped off from the silica gel plate, transmethylated and thereafter analyzed in a gas chromatograph.

Hydrolysis buffer

0.1 M	boric acid, pH 8.0
3 mM	$CaCl_2$
1.4 mM	sodium deoxycholate

f) Transmethylation of fatty acids with sodium methylate (method of Lühs)

After the solvent had been evaporated, or after material had been scraped from the thin-layer plate (for example in the case of *sn*-2 analysis of the total lipids), lipid samples were treated with 2 ml of sodium methylate solution for transesterification purposes. The mixture was shaken thoroughly and, in order to subject the fatty acids to transmethylation, incubated for approximately 30 minutes at room temperature. Thereafter, 1.5 ml of isooctane were added and the samples were carefully shaken twice. The mixture was stored for 30 minutes at 4°C, during which time the fatty acid methyl esters (FAMEs) enter the isooctane phase. After clear phase separation had occurred, the top phase, which was the isooctane phase, was pipetted into a GC tube and the sample was analyzed in a gas chromatograph.

Sodium methylate solution

5 g of sodium methylate were dissolved in 800 ml of methanol (99%) at 50°C, using a magnetic stirrer, and, after cooling, made up to 1000 ml with isooctane.

g) Methylation of free fatty acids with methanolic sulfuric acid

In a Pyrex tube with screw top, 1 ml of 1 N methanolic sulfuric acid was added to the concentrated lipid extract. The mixture was incubated for one hour at 80°C. After the mixture had been cooled briefly, it was treated with 1 ml of 0.9% NaCl and mixed. Thereafter, an equal volume of hexane was added, and the mixture was mixed thoroughly and incubated at 4°C for 30 minutes until phase separation took place. The hexane phase, which was the top phase, was transferred into a GC tube and analyzed in a gas chromatograph.

Methanolic sulfuric acid

2 ml of dimethoxypropanes and 0.5 M H_2SO_4 were added to 100 ml of (anhydrous) methanol.

h) Gas-chromatographic analysis

The following parameters of the gas-chromatographic system were maintained for the GC analyses:

Equipment type	HP 6890 GC
Injector	HP GC injector
Detector	flame ionization detector (FID), temp. 250°C
Column	J&W DW23 50% cyanopropyl/methylsiloxanes, 30 m, 0.5 mm diameter
Oven temperature	220°C
Carrier gas	hydrogen
Autosampler	HP 7673, injection volume 1 µl of sample

i) The lipid analysis of the Thraustochytrium lipids gave the following results

Lipid fraction	Fatty acid composition			
	16:0	22:3 ω -3	22:4 ω -3	22:6 ω -3
Total TAG	24%	12%	31%	23%
TAG sn-2	21%	26%		43%
Total membrane lipids	16%	13%		23%
Membrane lipids sn-2	34%	18%		36%

The results show that Thraustochytrium has a high DHA content in its lipids. With besides palmitate, DHA is the main component of the triacylglyerols and dominating fatty acid of the membrane lipids. It is noticeable that DHA is markedly concentrated at the sn-2 position of both the triacylglycerol and the membrane lipids: 36-43% of the fatty acids at the sn-2 position is DHA. As a result of this data, it can be assumed that Thraustochytrium has an active LPAAT with a high specificity for DHA-CoA.

Example 8: Isolation of total RNA and poly(A)$^+$ RNA

Total RNA was isolated from plants such as linseed and oilseed rape etc. by a method described by Logemann et al. (Anal. Biochem. (1987) 163: 21). The total RNA can be obtained from the moss Physcomitrella patens from protonemal tissue using the GTC method (Reski et al. (1994) Mol. Gen. Genet. 244: 351-359).

a) RNA isolation from Thraustochytrium, Cryptecodinium and Shewanella:

Frozen algal samples (-70°C) were comminuted in an ice-cold mortar under liquid nitrogen to give a fine powder. 2 volumes of homogenization medium (12.024 g sorbitol, 40.0 ml 1 M Tris-RC1, pH 9 (0.2 M); 12.0 ml 5 M NaCl (0.3 M), 8.0 ml 250 mM EDTA, 761.0 mg EGTA, 40.0 ml 10% SDS were made up to 200 ml with H_2O and the pH was brought to 8.5) and 4 volumes of phenol comprising 0.2% of mercaptoethanol were added to the frozen cell powder at 40-50°C, with thorough mixing. Thereafter, 2 volumes of chloroform were added and the mixture was stirred vigorously for 15 minutes. The mixture was centrifuged for 10 minutes at 10 000 g and the aqueous phase was extracted with phenol/chloroform (2 vol/2 vol) and finally with chloroform.

The resulting volume of the aqueous phase was treated with 1/20 vol of 4 M sodium acetate (pR 6) and 1 vol of isopropanol (ice-cold), and the nucleic acids were precipitated ON (= overnight) at -20°C. The mixture was centrifuged for 30 minutes at 10 000 g and the supernatant was pipetted off. This was followed by a wash step with 70% EtOH and another centrifugation. The sediment was in Tris borate buffer (80 mM Tris borate buffer, 10 mM EDTA, pH 7.0). Then, the supernatant was mixed with 1/3 vol of 8 M LiCl, mixed and incubated for 30 minutes at 4°C. After recentrifugation, the sediment was washed with 70% ethanol and centrifuged, and the sediment was subsequently dissolved in RNAse-free water.

Poly(A}+ RNA was isolated using Dyna Beads (Dynal, Oslo, Finland) following the instructions in the manufacturer's protocol.

After the RNA or poly(A}+ RNA concentration had been determined, the RNA was precipitated by addition of 1/10 volume of 3 M sodium acetate, pH 4.6, and 2 volumes of ethanol and stored at -70°C.

For the analysis, in each case 20 µg of RNA were separated in a formaldehyde-comprising, 1.5% strength agarose gel and transferred onto nylon membranes

(Hybond, Amersham). Specific transcripts were detected as described by Amasino (Amasino (1986) Anal. Biochem. 152: 304).

Example 9: Construction of cDNA libraries

To construct the cDNA libraries from Physcomitrella, Thraustochytrium and Fusarium, the first-strand synthesis was carried out using reverse transcriptase from murine leukemia virus (Roche, Mannheim, Germany) and oligo-d(T) primers, while the second-strand synthesis was achieved by incubation with DNA polymerase I, Klenow enzyme and RNAse H cleavage at 12°C (2 hours), 16°C (1 hour) and 22°C (1 hour): the reaction was stopped by incubation at 65°C (10 minutes) and subsequently transferred onto ice. Double-stranded DNA molecules were made blunt-ended using T4 DNA polymerase (Roche, Mannheim) at 37°C (30 minutes). The nucleotides were removed by means of phenol/chloroform extraction and Sephadex G50 centrifugation columns. EcoRI/XhoI adapters (Pharmacia, Freiburg, Germany) were ligated onto the cDNA ends by means of T4 DNA ligase (Roche, 12°C, overnight), cut again with XhoI and phosphorylated by incubation with polynucleotide kinase (Roche, 37°C, 30 min). This mixture was subjected to separation on a low-melting agarose gel. DNA molecules of above 300 base pairs were eluted from the gel, extracted with phenol, concentrated on Elutip D columns (Schleicher and Schüll, Dassel, Germany) and ligated with vector arms and packaged in lambda-ZAPII phages or lambda-ZAP Express phages using the Gigapack Gold kit (Stratagene, Amsterdam, the Netherlands), using the manufacturer's material and following their instructions.

Example 10: DNA sequencing and computer analysis

cDNA libraries as described in example 9 were used for DNA sequencing by standard methods, in particular by means of the chain termination method using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer, Weiterstadt, Germany). Random individual clones were sequenced after plasmid preparation from cDNA libraries via in-vivo mass excision and retransformation of DH10B on agar plates (details on materials and protocol from Stratagene, Amsterdam, the Netherlands). Plasmid DNA was prepared from E. coli overnight cultures which had been grown in Luria broth with ampicillin (see Sambrook et al. (1989) (Cold Spring Harbor Laboratory Press: ISBN 0-87969-309-6)) on a Qiagen DNA preparation robot (Qiagen, Hilden) following the manufacturer's protocol. Sequencing primers with the following nucleotide sequences were used:

5'-CAGGAAACAGCTATGACC-3'

5'-CTAAAGGGAACAAAAGCTG-3'

5'-TGTAAAACGACGGCCAGT-3'

The sequences were processed and annotated using the standard software package EST-MAX, which is commercially available from Bio-Max (Munich, Germany). Using

comparative algorithms, and using a search sequence, the BLAST program was used for searching for homologous genes (Altschul et al. (1997) "Gapped BLAST and PSI-BLAST: A new generation of protein database search programs", Nucleic Acids Res. 25: 3389-3402).

Example 11: Identification of genes by means of hybridization

Gene sequences can be used for identifying homologous or heterologous genes from cDNA or genomic libraries.

Homologous genes (i.e. full-length cDNA clones which are homologous, or homologs) can be isolated via nucleic acid hybridization using, for example, cDNA libraries: depending on the frequency of the gene of interest, 100 000 up to 1 000 000 recombinant bacteriophages are plated and transferred onto a nylon membrane. After denaturation with alkali, the DNA is immobilized on the membrane, for example by UV crosslinking. Hybridization is effected under high-stringency conditions. The wash steps and the hybridization are carried out in aqueous solution at a ionic strength of 1 M NaCl and a temperature of 68°C. Hybridization probes were prepared for example by labeling by means of radioactive (32P) nick transcription (High Prime, Roche, Mannheim, Germany). The signals are detected by means of autoradiography.

Partially homologous or heterologous genes which are related, but not identical, can be identified analogously to the above-described method using low-stringency hybridization and wash conditions. The ionic strength for the aqueous hybridization was usually kept at 1 M NaCl, the temperature being lowered gradually from 68 to 42°C.

Gene sequences with homologies with only a single domain of, for example, 10 to 20 amino acids can be isolated using synthetic radiolabeled oligonucleotide probes. Radiolabeled oligonucleotides are prepared by phosphorylating the 5′ end of two complementary oligonucleotides with T4 polynucleotide kinase. The complementary oligonucleotides are hybridized with one another and ligated so that concatemers are formed. The double-stranded concatemers are radiolabeled, for example by Nick transcription. Hybridization is usually effected under low-stringency conditions, using high oligonucleotide concentrations.

Oligonucleotide hybridization solution:

6 x SSC
0.01 M sodium phosphate
1 mM EDTA (pH 8)
0.5% SDS
100 µg/ml denatured salmon sperm DNA
0.1% dry skim milk

During the hybridization, the temperature was gradually reduced to 5-10°C below the calculated oligonucleotide Tm or down to room temperature means RT = 23°C in all

experiments, unless otherwise specified), followed by wash steps and autoradiography. Washing was carried out with extremely low stringency, for example 3 wash steps using 4 x SSC. Further details are as described by Sambrook, J., et al. (1989), "Molecular Cloning: A Laboratory Manual", Cold Spring Harbor Laboratory Press, or Ausubel, F.M., et al. (1994) "Current Protocols in Molecular Biology", John Wiley & Sons.

Example 12: Isolation and cloning of an LPAAT full-length clone from Thraustochytrium

Screening a Thraustochytrium cDNA library

Analogously to what has been described for example 9, a Thraustochytrium cDNA library was generated. In the next step, the phage library was converted into a plasmid library by means of a helper phage, following the manufacturer's instruction. The plasmid library was plated on LB medium, 0.8% agar, 100 mg/l ampicillin and incubated. Grown bacterial colonies were selected randomly, grown in liquid medium (LB, 100 mg/l ampicillin) and sequenced as described in example 10.

The sequences obtained were searched for redundancies, and these were removed. This gave rise to an assortment of sequences which describes a unigene set. This sequence set was input into the Pedant database (Biomax AG, Martinsried, Germany). A short sequence section with a low degree of similarity to known acyltransferases was found by means of BLAST analysis, using conserved regions within acyltransferases. The existing sequence information was used for generating primers (LPAAT069-5' and LPAAT069-3'). Using this fragment, the cDNA library was then searched for a full-length clone (table 8).

Table 8: Sequences of the primers employed

The melting point T_m (°C) of the oligonucleotides was calculated by the method of Suggs et al. (1981): T_m (°C) = 4 (G+C) + 2 (A+T) T_m values in brackets refer to actually binding nucleotides of primers whose ends have been modified by additionally introduced cleavage sites.

Primer	Sequence	T_m (°C)
LPAAT069-5´	5'-GCT ACA TTG CCA TGG AGC–3'	56
LPAAT069-3´	5'-GCT ACA AGA GGT CAG GTC G–3'	59
ACtrau-5´	5'-CTG GAT CCA TGA GCG CGT GGA CGA G–3'	69 (52)
ACtrau-3´	5'-TTG GAT CCC AAG AGG TCA GGT CGG A–3'	66 (54)
ACtrau-3´stop	5'-TTG GAT CCC TAC AAG AGG TCA GGT CG–3'	66 (48)
YES-HIS-5´	5'-CTG AGC TCA TGA GCG CGT GGA G–3'	69 (56)
YES-HIS-3´	5'-ATG GAT CCG TGA TGG TGA TGG TGA TGC AAG AGG TC–3'	72 (40)

In the PCR experiments, the constituents of a PCR standard mix, shown hereinbelow, were pipetted into a PCR reaction vessel on ice, placed into the thermoblock, and the temperature profile shown hereinbelow was started. The polymerase employed was in almost all cases Taq polymerase (Gibco BRL), with Pfu polymerase (Stratagene) only being used for amplifications for the purposes of functional expression in E. coli JC201. In all experiments, the polymerase was added via what is known as a "hot start", where the enzyme is added only after the DNA template has been subjected to denaturation for 5 minutes. The annealing temperatures (T_a) were chosen to be 3-5°C below the mean melting point T_m of the primer pairs.

PCR standard mix (for Taq polymerase)

5 µl 10 x PCR buffer (100 mM Tri-HCl, pH 8.3; 15 mM $MgCl_2$, 500 mM KCl)
1 µl dNTP mix (10 mM dATP, dGTP, dTTP and dCTP)
1 µl primer 1 (30 µM)
1 µl primer 2 (30 µM)
1 U Taq polymerase
50-100 ng plasmid DNA template
make up to 50 µl with distilled water

Hot-start program

1. denaturation 95°C, 5 min
2. hot start 25°C, 3 min → addition of the polymerase
3. denaturation 94°C 30 s
4. annealing T_m-5°C, 30 s
5. polymerization 72°C, 1 - 3 min (approx. 60 s for 1.0 kbp)

Steps 3. to 5. were repeated cyclically 25 to 30 times.

6. polymerization 72°C, 5 min
7. termination 4°C

a) Cold labeling of DNA

DNA probes were cold-labeled using the "PCR DIG PROBE SYNTHESIS KIT" (Boehringer Mannheim). To do so, DNA fragments were labeled in a PCR reaction with digoxigenin-labeled deoxyuridine triphosphate (DIG-dUTP). The detection was subsequently carried out by means of an anti-digoxygenin antibody which is conjugated with alkaline phosphatase, and addition of chemiluminescence or color substrates.

To avoid background signals which can be attributed to vector sequences, the PCR labeling first involved, in a first PCR, the amplification of the desired DNA with unlabeled dNTPs, the linear fragment was purified via an agarose gel and used as template for the actual PCR labeling, in which, in turn, the primer pair of the first PCR was employed. The labeling reaction was carried out as specified in the manufacturer's instructions. The chosen primer combinations are compiled in the table which follows.

Primer	Sequence
LPAAT069-5´	5'- GCT ACA TTG CCA TGG AGC –3'
LPAAT069-3´	5'- GCT ACA AGA GGT CAG GTC G –3'

b) Screening a cDNA library

To isolate a complete clone, a Thraustochytrium cDNA library (in λTriplEx2) was searched with the DIG-labeled probe. The probe was generated using the primers LPAAT069-3´ and LPAAT069-5, derived from the EST clone s_t002038069 known cDNA sequence which might code for a Thraustochytrium LPAAT.

5×10^4 plaques were plated in each case on 10 large NZY plates, following the manufacturer's instructions (Stratagene). To transfer the phages onto nitrocellulose filters (Hybond™-C, Amersham), the filters were placed on the plates for 1 minute, and their precise position was marked by 3 stamps with a cannula. The filters, stamped side uppermost, were subsequently treated first for 5 minutes with denaturation solution, then for 5 minutes with neutralization solution and finally for 15 minutes with 2 x SSC

solution. This was carried out using 3 sheets of Whatman 3 MM paper which had been impregnated with the solutions. After the filters had dried for 5 minutes, the DNA was immobilized by UV treatment with 0.12 Joule/cm^2 (UV-Crosslinker, Hoefer Scientific Instruments). Hybridization and colorimetric detection were carried out using the "Dig System für Filter Hybridisierung" from Boehringer (Mannheim) in accordance with the manufacturer's instructions. The hybridization buffers used were standard buffers, the hybridization being carried out in 80 ml of hybridization buffer using 15 µl of the probe PCR mix. After detection had been effected, the precise position of the signals and the three reference points of the filters were transferred to plastic films in order to identify the positive plaques on the plates, using the former as stencil. The positive plaques were then excised using a flamed cork borer (diameter 5 mm), transferred into 1 ml of SM buffer supplemented with 20 µl of $CHCl_3$, and the phages were eluted from the agar plugs overnight at 4°C. Accurate excision of the plaques was almost impossible as the result of their high density and small size. This is why, as a rule, one to two rescreens are carried out. In this case, the phage lysates were studied for approx. 570 bp fragments by means of PCR and the primers LPAAT069-3′ and LPAAt-5. To this end, aliquots of the phage lysates were treated with EDTA (final concentration 10 mM), and 1 µl of this was employed as template for the PCR. Using positive lysates, in-vivo excisions were carried out as specified in the "ZAP-cDNA® Gigapack® II Gold Cloning Kit" (Stratagene), but instead of the 10-50 µl as stated, only 2 µl of the infected SOLR cells were plated onto LB-Amp plates and incubated overnight at 37°C. The plasmids from the resulting colonies were analyzed directly by means of PCR and the primers LPAAT-3′ and LPAAT-5′. To this end, pools were generated by rubbing in each case 6 colonies into 20 µl of distilled water in an Eppendorf tube, using sterile toothpicks, and the tubes were subjected to 3 x freeze-thaw cycles in order to lyze the cells, centrifuged for 5 minutes at 14 000 x g, and 2 µl of the supernatant was employed as template in the PCR reaction. Positive pools were isolated, and the plasmids were isolated via plasmid minipreps and analyzed via PCR, restriction analyses and DNA sequencing reactions.

Finally, a Thraustochytrium LPAAT full-length clone was identified; its DNA sequence is shown in SEQ ID NO: 1. The derived amino acid sequence is shown in SEQ ID NO: 2.

NZY medium (per liter, NZY plates made with 15 g agar)

5 g NaCl
5 g yeast extract
10 g NZ amine (casein hydrolysate)
pH 7.5 (NaOH)
2 g $MgSO_4$ (filter-sterilized)

Denaturation solution

0.5 M NaOH
1.5 M NaCl

Neutralization solution

1.0 M Tris-HCl, pH 7.5
1.5 M NaCl

20 x SSC

3.0 M NaCl
0.3 M sodium citrate, pH 7.0

Standard buffer

5 x SSC
0.1% (w/v) N-laurylsarcosine
0.02% (w/v) SDS
1% blocking reagent

SM buffer (per liter)

5.8 g NaCl
2 g $MgSO_4$
50 ml 1 M Tris-HCl, pH 7.5
5 ml 2% strength gelatin

Example 13: Isolation and cloning of full-length clones for PUFA-specific acyltransferases from Physcomitrella patens, Mortierella alpina and Shewanella hanedai

RNA was isolated, and a cDNA library generated, from Physcomitrella patens and Mortierella alpina as described in examples 8 and 9.

In the next step, the phage library was converted into a plasmid library by means of a helper phage, following the manufacturer's instructions. The plasmid library was plated on LB medium, 0.8% agar, 100 mg/l ampicillin and incubated. Grown bacterial colonies

were selected randomly, grown in liquid medium (LB, 100 mg/l ampicillin) and sequenced as described in example 10.

The sequences obtained were searched for redundancies, and these were removed. This gave rise to an assortment of sequences which describes a unigene set. This sequence set was input into the Pedant database (Biomax AG, Martinsried, Germany). Short sequence sections with a low degree of similarity to known acyltransferases were found by means of BLAST analysis, using conserved regions within acyltransferases (table 9). The existing sequence information was used for generating primers (table 10). Using these primers, the full-length clone was amplified.

For the Shewanella hanedai acyltransferase, the public database of Shewanella putrefaciens MR1 (TIGR database http://tigrblast.tigr.org/ufmg/) was searched for acyltransferases. A sequence with homology to acyltransferases was found in the database. A PCR fragment of this sequence was generated by means of standard primers T7 and T3. The resulting product was illustrated as in example 10 a) and b), labeled and employed for searching a genomic Shewanella hanedai library.

Shewanella hanedai genomic DNA was isolated by the following protocol:
A 100 ml culture was grown at 30°C to an optical density of 1.0. 60 ml of this were centrifuged for 3 minutes at 3000 x g. The pellet was resuspended in 6 ml of twice-distilled H_2O and divided between 1.5 ml vessels, centrifuged, and the supernatant was discarded. The pellets were resuspended and lyzed by vortexing with 200 µl of solution A, 200 µL of phenol/chloroform (1:1) and 0.3 g of glass beads. After addition of 200 µl of TE buffer pH 8.0, the mixture was centrifuged for 5 minutes. The supernatant was subjected to ethanol precipitation with 1 ml of ethanol. After the precipitation, the resulting pellet was dissolved in 400 µl of TE buffer pH 8.0 + 30 µg/ml Rnase A. After incubation for 5 minutes at 37°C, 18 µl of 3 M sodium acetate solution pH 4.8 and 1 ml of ethanol were added, and the precipitated DNA was pelleted by centrifugation. The DNA pellet was dissolved in 25 µl of twice-distilled H_2O. The concentration of the genomic DNA was determined by its absorption at 260 nm.

Solution A:
2% Trition-X100
1% SDS
0.1 M NaCl
0.01 M Tris-HCl pH 8.0
0.001 M EDTA

The resulting genomic DNA was incubated with the restriction enzyme Sau3A (New England Biolabs) for 1 hour at 25°C following the manufacturer's instructions. The resulting fragments were then ligated into a BamHI-digested pUC18 plasmid, using T4 ligase (Roche). The resulting library was then searched in the same manner as described in example 10. A clone comprising a 1.7 kb genomic fragment and having a 687 bp coding sequence with similarity to acyltransferases was found.

The Shewanella hanedai sequence has a particularly high degree of similarity to the Chaenorabdidis elegans LPCAT. The similarity of the two sequences at the amino acid level is 26%.

Table 9: Identified acyltransferase from the abovementioned cDNA libraries

Clone No.	**Organism**	**Homology with**
MaLPAAT1.1	M. alpina	LPAAT
MaLPAAT1.2	M. alpina	LPAAT
ShLPAAT	S. hanedai	LPAAT
T6	Thrausto.	LPAAT
pp004064045r	P. patens	LPAAT
pp020064227r	P. patens	LPAAT
pp015052144r	P. patens	GPAT/LPAT
pp004034225r	P. patens	GPAT
pp004104272r	P. patens	Ca-LPAAT
pp020018156r	P. patens	Ca-LPAAT
pp015034341r	P. patens	LPAAT
pp015033362r	P. patens	LCAT
Fg003028298	Fusarium	LCAT

Table 10: Sequences of the primers employed:

Clone No.	Organism	Primer sequence in 5'-3' orientation	Length in bp
MaLPAAT1.1	M. alpina	atggatgaatccaccacgacca tcagcccgatgcttgctgc	1254
MaLPAAT1.2	M. alpina	atgaaccctatctacaagggt tcagcccgatgcttgctgc	1170
ShLPAAT	S. hanedai	atgttactgctagcatttgt ttactttgccattaagg	687
T6	Thrausto.	atgagcgcgtggacgagggc ctacaagaggtcaggtcggacgtaca	918
Pp00406404	P. patens	Atggctttgatgtatatctg ttacacgatttttcttttag	714
Pp02006422	P. patens	atgctgatattacagcccttc ctaatgaacaggaagaccgt	657
Pp01505214	P. patens	atgatccggattttcagag tcagtccgttttgccgaggt	444
Pp00403422	P. patens	atgccgtcgctgtttcggg tcaatcagttcgcctgcttc	1305
Pp00410427	P. patens	atgctgatattacagcccttc ctaatgaacaggaagaccgt	1566
Pp02001815	P. patens	atgaccagcacggaaaatac ctagatgttagtttcactc	1560
Pp01503434	P. patens	atgattatgatggaggtgctg tcagtccgttttgccgagg	1014
Pp01503336	P. patens	atgtgttcaatttcttgtgg ttagtggaacataagctgtt	1503
Fg003028298	Fusarium	atgggaaagtccactttac ctatgaagtctcctcatcatcg	1893

In the PCR experiments, the constituents of a PCR standard mix, shown hereinbelow, were pipetted into a PCR reaction vessel on ice, placed into the thermoblock, and the temperature profile shown hereinbelow was started. The polymerase employed was in almost all cases Taq polymerase (Gibco BRL), with Pfu polymerase (Stratagene) only being used for amplifications for the purposes of functional expression in E. coli JC201. In all experiments, the polymerase was added via what is known as a "hot start", where the enzyme is added only after the DNA template has been subjected to denaturation for 5 minutes. The annealing temperatures (T_a) were chosen to be 3-5°C below the mean melting point T_m of the primer pairs.

PCR standard mix (for Taq polymerase)

5 µl 10 x PCR buffer (100 mM Tri-HCl, pH 8.3; 15 mM $MgCl_2$, 500 mM KCl)
1 µl dNTP mix (10 mM dATP, dGTP, dTTP and dCTP)
1 µl primer 1 (30 µM)
1 µl primer 2 (30 µM)
1 U Taq polymerase
50-100 ng plasmid DNA template
make up to 50 µl with distilled water

Hot-start program

1. denaturation 95°C, 5 min
2. hot start 25°C, 3 min → addition of the polymerase
3. denaturation 94°C 30 s
4. annealing T_m-5°C, 30 s
5. polymerization 72°C, 1 - 3 min (approx. 60 s for 1.0 kbp)

Steps 3. to 5. were repeated cyclically 25 to 30 times.

6. polymerization 72°C, 5 min
7. termination 4°C

GSP: TCT CTT TTT CGT GCT GCT CCA GCC GAT (Are 297)

PCR program: 10 min. 95°C

1 min. 95°C (40 cycles)
1 min. 65°C
2 min. 72°C
10 min. 72°C interval 4°C

PCR apparatus: Biometra Trio Thermoblock

First PCR on the RACE library moss with AP1 and GSP, when size correct PCR with nested AP2 and GSP, positives are cloned into pCRII-TOPO-TA cloning vector for sequencing purposes.

Example 14: Expression of Thraustochytrium LPAAT (ThLPAAT) in yeast

To detect the functionality of ThLPAAT, the coding region of the cDNA was, in a first approach, cloned into a yeast expression vector and expressed in S. cerevisiae. The LPAAT produced in the yeast should be detected added via acyltransferase activity in microsomal fractions.

All solid and liquid media for yeast were prepared by protocols of Ausubel et al. (Current Protocols in Molecular Biology, John Wiley & Sons, New York, 1995).

The ThLPAAT cDNA was excised from the vector pGEM-T by a restriction digest with HindIII/BamHI, cloned into the HindIII/BamHI-cut shuttle vector pYES2 (Invitrogen, Carlsbad, USA), and the resulting vector pYES2-ThLPAAT was transformed into E. coli XL1 Blue. With the aid of the LiAc method, pYES2-ThLPAAT was transformed into S. cerevisiae INCSc1 (Invitrogen, Carlsbad, USA), where the expression of the ThLPAAT cDNA was under the control of the GAL1 promoter.

The expression of ThLPAAT in S. cerevisiae INVSc1 was carried out by a modified method of Avery et al. (Appl. Environ. Microbiol., 62, 1996: 3960–3966) and Girke et al. (The Plant Journal, 5, 1998: 39–48). To prepare a starter culture, 20 ml of SD medium supplemented with glucose and amino acid solution, but without histidine, were inoculated with an individual yeast colony and incubated overnight at 30°C at 140 rpm. The cell culture was washed twice by centrifugation and resuspended in SD medium without supplements and without sugar. The washed cells were used to inoculate a main culture to an OD_{600} of from 0.1 to 0.3. The main culture was grown in 25 ml of SD medium supplemented with 2% (w/v) galactose, amino acid solution without histidine, 0.02% linoleic acid (2% strength stock solution in 5% Tergitol NP40), 10% Tergitol NP40 for 72 hours at 30°C. The main culture was harvested by centrifugation. The cell pellet was frozen at -20°C and then lyophilized for approximately 18 hours.

After expression of the construct pYES2-ThLPAAT in yeast, no active protein was purified, nor did the subcellular fractions from the different transgenic cells show higher LPAAT activities than the corresponding control fractions.

To increase the solubility of the expressed protein, a further construct pDest15-GST-ThLPAAT (pDest15 vektor from Invitrogen) was generated via the Gateway reaction. To this end, the following primers were synthesized following the manufacturer's instructions:

5' primer att1ThLPAAT:

GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGAGCGCGTGGACGAGGGCC

3' primer att2ThLPAAT:

GGGGACCACTTTGTACAAGAAAGCTGGGTCTAGTGGTGGTGGTGGTGGTGCAAG AGGTCAGGTCGGACGTAC

These primers were used to carry out the following PCR reaction:

PCR standard mix (for Taq polymerase)

5 µl 10 x PCR buffer (100 mM Tri-HCl, pH 8.3; 15 mM $MgCl_2$, 500 mM KCl)
1 µl dNTP mix (10 mM dATP, dGTP, dTTP and dCTP)
1 µl primer 1 (30 µM)
1 µl primer 2 (30 µM)
1 U Taq polymerase
50-100 ng pYES2-ThLPAAT
make up to 50 µl with distilled water

PCR program: 2 min. 95°C

1 min. 95°C	(30 cycles)
1 min. 65°C	
2 min. 72°C	
10 min. 72°C	interval 4°C

PCR apparatus: Biometra Trio Thermoblock

The PCR product was transferred into the vector pDONOR221 by Gateway reaction (BP reaction; Invitrogen) following the manufacturer's instructions, and the sequence was verified by sequencing. In a next step, the ThLPAAT sequence was then transferred into the vector pDES15 by the LR reaction and employed for expression in E. coli BL21 cells. The ThLPAAT sequence was attached to the open reading frame of the glutathione-S transferase (GST) encoded in the plasmid, in accordance with the manufacturer's instructions. This gave rise to a fusion protein of GST and ThLPAAT.

Expressed protein was detected after expression under standard conditions in E. coli (fig. 21A) and purified via a glutathione column.

The purified fusion protein showed LPAAT activity, as shown in fig. 21B. The highest activity was obtained for DHA-CoA (22:6), which makes possible a utilization of this acyltransferase for the production of PUFA.

Figure 21A shows the Western blot analyses of the *Thraustochytrium* LPAAT expressed in *E. coli* as fusion protein (LPAAT-FP) with N-terminal GST tag and C-terminal His tag (lines E: 7 µg soluble protein fraction, line M: size standard).
Figure 21B shows the acyl-CoA specificity of the *Thraustochytrium* LPAAT, expressed as GST fusion protein, in *E. coli*. The enzyme assays were determined using 0.4 µg of soluble protein fraction in the presence of 100 mM Tricine-NaOH (pH 8.2), 30 µM 1-oleoyl[U-^{14}C]glycerol-3-phosphate and increasing concentrations of the thioesters detailed.

Example 15: Expression of Shewanella LPAAT

To clone an LPAAT gene from the prokaryotic organism *Shewanella*, the genomic DNA from *Shewanella hanedai* was isolated, partially digested with Sau3a and ligated into the vector pUC18. This genomic library was screened for LPAAT genes by a PCR using different primer combinations. This method has made it possible to identify a 1486 bp clone whose open reading frame codes for a 25.2 kDa LPAAT protein. The ShLPAAT sequence was introduced into the vector pQE70 (Qiagen) in accordance with the manufacturer's instructions. The resulting plasmids pQE70-Sh and pQE70-ShHis and the blank vector pQE70 were transformed into E. coli BL21 cells and expressed at 10°C (figure 22A). Active protein was obtained at this temperature only (figure 22B). The membrane fractions were used for this purpose in the further experiments. In both expression forms, this fraction showed a high level of activity with regard to the incorporation of DHA-CoA (22:6-CoA). The high incorporation rate with regard to PUFA acyl-CoA residues is required for the use for the production of PUFA.

Figure 22**A:** shows the Western blot analysis of the *Shewanella* LPAAT expressed in *E. coli* as fusion protein with C-terminal His-tag (line E: 7 µg of inclusion body fraction, line F: 7 µg of membrane fraction, line M: size standard). Figure 22**B:** shows the functional expression of the *Shewanella* LPAAT in *E. coli* enzyme assays. The assays were carried out with extracts (1 µg) from *E. coli* comprising the blank vector (pQE70) or a Shewanella construct without (pQE-Sh) or with His-Tag sequence at the 3' end (pQE-ShHis) in the presence of 30 µM 1-oleoyl[U-^{14}C]glycerol-3-phosphate and 30 µM of the detailed thioesters.

Example 16: Expression of Mortierella LPAAT (MaLPAAT, MaB4) in yeast

The MaLPAAT cDNA was amplified via PCR with the stated primers MaLPAAT2.1, the PCR product was cloned into the vector pENTR-SD-D-TOPO (Invitrogen, Carlsbad, USA) in accordance with the manufacturer's instructions and transformed into E. coli XL1 Blue. The MaLPAAT fragment was transferred from the resulting vector pENTR-SD-D-MaLPAAT via Gateway reaction in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, USA) into the vector pYES54Dest, resulting in the vector pYES52Dest-MaLPAAT. PYES52Dest-MaLPAAT was transformed into S. cerevisiae INCSc1 (Invitrogen, Carlsbad, USA) with the aid of the LiAc method.

Yeast cells which had been transformed with the plasmid pYES52Dest-MaLPAAT were analyzed as follows:

Yeast colonies which, after transformation, were capable of growing on dropout uracil minimal medium were again streaked on dropout uracil minimal medium and then grown on liquid minimal medium to an OD600 of 0.8. This preculture was then used for inoculating the main culture which, besides the minimal medium, additionally comprised 2% (w/v) galactose and 250 µM of the fatty acids. After incubation of the main culture for 24 hours at 30°C, the cells were harvested by centrifugation (100 x g, 10 min, 20°C) and washed with 100 mM $NaHCO_3$, pH 8.0, in order to remove residual

medium and fatty acids. Fatty acid methyl esters (FAMEs) were prepared from the yeast cell sediments by acid methanolysis. To this end, the cell sediments were incubated for 1 hour at 80°C with 2 ml of 1N methanolic sulfuric acid and 2% (v/v) dimethoxypropane. The FAMEs were extracted by two extractions with petroleum ether (PE). To remove nonderivatized fatty acids, the organic phases were washed in each case once with 2 ml of 100 mM $NaHCO_3$, pH 8.0, and 2 ml of distilled water. Thereafter, the PE phases were dried with Na_2SO_4, evaporated under argon and taken up in 100 µl of PE. The samples were separated on a DB-23 capillary column (30 m, 0.25 mm, 0.25 µm, Agilent) in a Hewlett Packard 6850 gaschromatograph equipped with flame ionization detector. The conditions for the GLC analysis were as follows: the oven temperature was programmed from 50°C to 250°C with an increment of 5°C/min and finally 10 minutes at 250°C (holding).

The signals were identified by comparing the retention times with corresponding fatty acid standards (Sigma).

The methodology is described for example in Napier and Michaelson, 2001, Lipids. 36(8):761-766; Sayanova et al., 2001, Journal of Experimental Botany. 52(360):1581-1585, Sperling et al., 2001, Arch. Biochem. Biophys. 388(2):293-298 and Michaelson et al., 1998, FEBS Letters. 439(3):215-218.

Figure 23 shows the results of the feeding experiments with the yeast cells which comprise plasmid pYES52Dest-MaLPAAT (MaB4_AT). In fig. 23, A/B, the yeast cultures were fed linoleic acid (18:2 Δ9,12). In comparison with the control culture (fig. 23, A), the yeast cells with the MaLPAAT showed a markedly higher conversion (increased 4-fold) of 18:2 into γ-linolenic acid (18:3 Δ6,9,12), and a 3.5-fold increase of the fatty acid 20:2 Δ11,14 elongated from 18:2. Analogously, when feeding linolenic acid (18:3 Δ9,12,15), a markedly higher conversion rate to give stearidonic acid (18:4 Δ6,9,12,15) and isoarachidonic acid (20:4 Δ8,11,14,17) was observed in comparison with the controls (figure 24).

Besides this activity, an enhanced conversion of 16:1 Δ9 (endogenous fatty acid in yeast) to give cis-vaccenic acid (18:1 Δ11) was observed in both feeding experiments.

Figure 25 and figure 26 show that the observed enhanced conversion rates of the substrates by the desaturase and the elongase also leads to an increase in the polyunsaturated fatty acids in the neutral lipid (oil). After the yeasts had been fed linoleic or linolenic acid, the yeast cells were extracted in chloroform:methanol (2:1) and applied to a silica thin-layer plate (Machery&Nagel, Düren). The thin-layer plate was incubated for 45 minutes with chloroform-methanol-H2O (65:25:4) in a chamber. In doing so, the neutral lipids (triacylglycerides) migrate with the solvent front. After the incubation had ended, the neutral lipids were scraped off from the plate, extracted with chloroform:methanol and analyzed by gas chromatography.

The increase in the conversion rate of PUFAs, which had been observed for the total extracts, was clearly also monitored in the neutral lipids. As regards the feeding of

linoleic acid (fig. 25 A and B), a 2-fold increase in the conversion of linoleic acid into γ-linolenic acid (18:3 Δ6,9,12) and a 3-fold increase in the 20:2 Δ9,12 content was observed. The feeding of linolenic acid (fig. 26, C and D) gave similar data (conversion of 18:3 into 18:4 3-fold, of 18:3 into 20:3 3-fold).

Thus, it was demonstrated that the increase in the PUFA content as the result of MaLPAAT leads to an increase in PUFAs in the oil (neutral lipids) of the yeasts.

Example 16: Plasmids for plant transformation

Binary vectors such as pBinAR can be used for transforming plants (Höfgen and Willmitzer (1990) Plant Science 66: 5221-230). The binary vectors can be constructed by ligating the cDNA in sense or antisense orientation into T-DNA. 5' of the cDNA, a plant promoter activates the transcription of the cDNA. A polyadenylation sequence is located 3' of the cDNA.

Tissue-specific expression can be achieved using a tissue-specific promoter. For example, seed-specific expression can be achieved by cloning the napin or the LeB4 or USP promoter 5' of the cDNA. Any other seed-specific promoter element can also be used. The CaMV-35S promoter can be used for obtaining constitutive expression in all of the plant. The expressed protein can be targeted into a cellular compartment using a signal peptide, for example for plastids, mitochondria or the endoplasmic reticulum (Kermode (1996) Crit. Rev. Plant Sci. 15: 285-423). The signal peptide is cloned 5' in the reading frame with the cDNA in order to obtain the subcellular localization of the fusion protein.

Example 17: Transformation of Agrobacterium

The Agrobacterium-mediated transformation of plants can be carried out for example using the Agrobacterium tumefaciens strain GV3101 (pMP90) (Koncz and Schell (1986) Mol. Gen. Genet. 204: 383-396) or LBA4404 (Clontech). The transformation can be carried out by standard transformation techniques (Deblaere et al. (1984) Nucl. Acids. Res. 13: 4777-4788).

Example 18: Plant transformation and expression of PUFA-specific acyltransferases in plants

The expression of LCPUFA-specific acyltransferases in transgenic plants is advantageous in order to increase the LCPUFA content in these plants. To this end, the acyltransferase cDNAs according to the invention were cloned into binary vectors and transferred into Arabidopsis thaliana, Nicotiana tabacum, Brassica napus and Linum usitatissimum via Agrobacterium-mediated DNA transfer. Here, the expression of the acyltransferase cDNA was under the control of the constitutive CaMV 35 S promoter or the seed-specific USP promoter.

Especially preferred in this context are transgenic plants which already express the desaturases and elongases required for the synthesis of LCPUFAs and which produce small amounts of these LCPUFAs.

The expression vectors used were the vector pBinAR (Höfgen and Willmitzer, Plant Science, 66, 1990: 221 - 230) or the pBinAR derivative pBinAR-USP, in which the CaMV 35 S promoter had been replaced by the V. faba USP promoter. The vectors pGPTV and pGPTV-USP were also used. To carry out the recloning step, it was necessary to excise the CalDes cDNA from the vector pGEM-T and clone it into pBinAR or pBinAR-USP. A further binary vector which was used was pSUN.

The resulting binary vectors with acyltransferase genes were transformed into Agrobacterium tumefaciens (Höfgen and Willmitzer, Nucl. Acids Res., 16, 1988: 9877). A. thaliana was transformed by means of floral dip (Clough and Bent, Plant Journal, 16, 1998: 735 - 743), and N. tabacum via coculturing tobacco leaf segments with transformed A. tumefaciens cells, and linseed and oilseed rape by coculturing hypocotyl segments with transformed A. tumefaciens cells.

The expression of the acyltransferase genes in transgenic Arabidopsis, tobacco, oilseed rape and linseed plants was analyzed via Northern blot analysis. Selected plants were analyzed for their content in punicic acid or other conjugated fatty acids such as CLA in the seed oil.

To obtain seed-specific expression of PuFADX and PuFAD12, it is also possible to use the napin promoter analogously to the USP promoter.

The Agrobacterium-mediated transformation of plants can be carried out using standard transformation and regeneration techniques (Gelvin, Stanton B., Schilperoort, Robert A., Plant Molecular Biology Manual, 2nd Ed., Dordrecht: Kluwer Academic Publ., 1995, in Sect., Ringbuc Zentrale Signatur: BT11-P ISBN 0-7923-2731-4; Glick, Bernard R., Thompson, John E., Methods in Plant Molecular Biology and Biotechnology, B. Raton: CRC Press, 1993, 360 S., ISBN 0-8493-5164-2).

For example, oilseed rape can be transformed by cotyledon or hypocotyl transformation (Moloney et al., Plant Cell Report 8 (1989) 238-242; De Block et al., Plant Physiol. 91 (1989) 694-701). The use of antibiotics for the selection of Agrobacteria and plants depends on the binary vector and the agrobacterial strain used for the transformation. Oilseed rape is usually selected using kanamycin as selectable plant marker. The agrobacterium-mediated gene transfer into linseed (Linum usitatissimum) can be carried out for example using a technique described by Mlynarova et al. (1994) Plant Cell Report 13: 282-285.

Soybean can be transformed for example using a technique described in EP-A-O 0424047 (Pioneer Hi-Bred International) or in EP-A-O 0397687, US 5,376,543, US 5,169,770 (University Toledo). The transformation of plants using particle bombardment, polyethylene glycol-mediated DNA uptake or via the silicon carbonate

fiber technique is described for example by Freeling and Walbot "The maize handbook" (1993) ISBN 3-540-97826-7, Springer Verlag New York).

Example 19: Analysis of the expression of a recombinant gene product in a transformed organism

The activity of a recombinant gene product in the transformed host organism was measured at the transcriptional and/or the translational level.

A suitable method for determining the amount of transcription of the gene (an indication of the amount of RNA available for the translation of the gene product) is to carry out a Northern blot as detailed hereinbelow (reference, see Ausubel et al. (1988) Current Protocols in Molecular Biology, Wiley: New York, or the abovementioned examples section), where a primer which is such that it binds to the gene of interest is labeled with a detectable label (usually a radioactive or chemiluminescent label) so that, when the total RNA of a culture of the organism is extracted, separated on a gel, transferred to a stable matrix and incubated with this probe, the binding, and the degree of the binding, of the probe indicates the presence and also the amount of the mRNA for this gene. This information indicates the degree of the transcription of the transformed gene. Cellular total RNA can be prepared from cells, tissues or organs using a plurality of methods, all of which are known in the art, such as, for example, the method described by Bormann, E.R., et al. (1992) Mol. Microbiol. 6:317-326.

Northern hybridization:

To carry out the RNA hybridization, 20 µg of total RNA or 1 µg of poly(A)$^+$ RNA were separated as described in Arnasino (1986, Anal. Biochem. 152, 304) by means of gel electrophoresis in agarose gels with a strength of 1.25% using formaldehyde, transferred by capillary attraction using 10 x SSC to positively charged nylon membranes (Hybond N$^+$, Amersham, Brunswick), immobilized by means of UV light and prehybridized for 3 hours at 68°C using hybridization buffer (10% dextran sulfate weight/vol., 1 M NaCl, 1% SDS, 100 mg herring sperm DNA). The DNA probe was labeled with the Highprime DNA labeling kit (Roche, Mannheim, Germany) during the prehybridization step, using alpha-32P-dCTP (Amersham, Brunswick, Germany). The hybridization was carried out at 68°C overnight in the same buffer after addition of the labeled DNA probe. The wash steps were carried out twice for 15 minutes using 2 X SSC and twice for 30 minutes using 1 X SSC, 1% SDS, at 68°C. The sealed filters were exposed at -70°C for a period of from 4 hours to 3 days.

To analyze the presence or the relative amount of protein translated by this mRNA, it is possible to employ standard techniques such as a Western blot (see, for example, Ausubel et al. (1988) Current Protocols in Molecular Biology, Wiley: New York). In this method, the cellular total proteins are extracted, separated by means of gel electrophoresis, transferred to a matrix such as nitrocellulose, and incubated with a probe, such as an antibody, which binds specifically to the desired protein. This probe is usually provided with a chemiluminescent or colorimetric label which is easy to

detect. The presence and the amount of the observed labeling indicates the presence and the amount of the desired mutated protein which is present in the cell.

Example 20: Analysis of the effect of the recombinant proteins on the production of the desired product

The effect of the genetic modification in plants, fungi, algae, ciliates, or on the production of a desired compound (such as a fatty acid) can be determined by growing the modified microorganisms or the modified plant under suitable conditions (like those described above) and analyzing the medium and/or the cellular components for the increased production of the desired product (i.e. of lipids or a fatty acid). These analytical techniques are known to the skilled worker and comprise spectroscopy,

thin-layer chromatography, various types of staining methods, enzymatic processes, microbiological processes and analytical chromatography such as high-performance liquid chromatography (see, for example, Ullmann, Encyclopedia of Industrial Chemistry, Vol. A2, pp. 89-90 and pp. 443-613, VCH: Weinheim (1985); Fallon, A., et al., (1987) "Applications of HPLC in Biochemistry" in: Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 17; Rehm et al. (1993) Biotechnology, Vol. 3, Chapter III:

"Product recovery and purification", pp. 469-714, VCH: Weinheim; Belter, P.A., et al. (1988) Bioseparations: downstream processing for Biotechnology, John Wiley and Sons; Kennedy, J.F., and Cabral, J.M.S. (1992) Recovery processes for biological Materials, John Wiley and Sons; Shaeiwitz, J.A., and Henry, J.D. (1988) Biochemical Separations, in: Ullmann's Encyclopedia of Industrial Chemistry, Vol. B3; Chapter 11, pp. 1-27, VCH: Weinheim; and Dechow, F.J. (1989) Separation and purification techniques in biotechnology, Noyes Publications).

In addition to the abovementioned processes, plant lipids are extracted from plant material as described by Cahoon et al. (1999) Proc. Natl. Acad. Sci. USA 96 (22) :12935-12940, and Browse et al. (1986) Analytic Biochemistry 152:141-145. Qualitative and quantitative lipid or fatty acid analysis is described by Christie, William W., Advances in Lipid Methodology, Ayr/Scotland: Oily Press (Oily Press Lipid Library; 2); Christie, William W., Gas Chromatography and Lipids. A Practical Guide - Ayr, Scotland: Oily Press, 1989, Repr. 1992, IX, 307 pp. (Oily Press Lipid Library; 1); "Progress in Lipid Research, Oxford: Pergamon Press, 1 (1952) -16 (1977) under the title: Progress in the Chemistry of Fats and Other Lipids CODEN.

Besides measuring the end product of the fermentation, it is also possible to analyze other components of the metabolic pathways which are used for the production of the desired compound, such as intermediate and secondary products, in order to determine the overall efficiency of the production of the compound. The analytical methods comprise measuring the amounts of nutrient in the medium (for example sugars, hydrocarbons, nitrogen sources, phosphate and other ions), measuring the biomass composition and the growth, analysis of the production of common

metabolites of biosynthetic pathways and measuring gases which are generated during the fermentation. Standard methods for these measurements are described in Applied Microbial Physiology; A Practical Approach, P.M. Rhodes and P.F. Stanbury, Ed., IRL Press, 10 pp. 131-163 and 165-192 (ISBN: 0199635773) and references cited therein.

One example is the analysis of fatty acids (abbreviations: FAMEs, fatty acid methyl esters; GC-MS, gas-liquid chromatography–mass spectrometry; TAG, triacylglycerol; TLC, thin-layer chromatography).

The unambiguous detection for the presence of fatty acid products can be obtained by means of analyzing recombinant organisms by analytical standard methods: GC, GC-MS or TLC, as described repeatedly by Christie and the references cited therein (1997, in: Advances on Lipid Methodology, Fourth Ed.: Christie, Oily Press, Dundee, 119-169; 1998, Gas-chromatography/mass spectrometry methods, Lipids 33:343-353).

The material to be analyzed can be disrupted by sonication, grinding in a glass mill, liquid nitrogen and grinding, or via other suitable methods. After disruption, the material must be centrifuged. The sediment is resuspended in distilled water, heated for 10 minutes at 100°C, cooled on ice and recentrifuged, followed by

extraction in 0.5 M sulfuric acid in methanol supplemented with 2% dimethoxypropane for 1 hour at 90°C, which leads to hydrolyzed oil and lipid compounds, which give transmethylated lipids. These fatty acid methyl esters are extracted in petroleum ether and finally subjected to GC analysis using a capillary column (Chrompack, WCOT Fused Silica, CP-Wax-52 CB, 25 micrometers, 0.32 mm) at a temperature gradient between 170°C and 240°C for 20 minutes and 5 minutes at 240°C. The identity of the resulting fatty acid methyl esters must be defined using standards which are available from commercial sources (i.e. Sigma).

In the case of fatty acids for which no standards are available, the identity must be shown via derivatization and subsequent GC-MS analysis. For example, the localization of fatty acids with triple bond must be shown via GC-MS after derivatization with 4,4-dimethoxyoxazoline derivatives (Christie, 1998, see above).

Equivalents

The skilled worker recognizes, or will find, a multiplicity of equivalents of the specific embodiments according to the invention described herein by simply using routine experiments. The patent claims are intended to encompass these equivalents.